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Flavor Chemistry of Wine and Other Alcoholic Beverages

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Michael C. Qian and Thomas H. Shellhammer

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Foreword

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Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

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Preface

Aroma is one of the most important quality attributes for wine and many other alcoholic beverages. However, the chemical composition of most alcoholic beverages is so complex that it has always been a challenge for scientists to fully understand their flavor chemistry. The low concentration of key aroma compounds, such as thiols, the low sensory threshold of many important contributors to aroma and the interfering alcohol matrix make the accurate analysis extremely challenging. With the advance of analytcial instrumentation, particularly the greater accessibility of LC-MS, new insights about the flavor and flavor precursors in wine and alcoholic beverages has been achieved.

This book is derived from the American Chemical Society symposium "Flavor Chemistry of Alcoholic Beverages" held on August 22-26, 2010, in Boston, MA, with the purpose of sharing new information on the flavor chemistry of wine, beer, and other other alcoholic berverages. Participants of this symposium were scientists from both the academic and industrial scientific communities.

A section of this book is devoted to the flavor and flavor precursors in wine grapes and their conversion in wine. This aspect is important because the origin of many unique aromas found in wine can be sourced directly to wine grapes. Since these aroma and aroma precursors are the secondary metabolites of plants, their biotransformation and accumulation are directly inflenced by environment and viticultural practice in the vineyeard.

Another significant portion focuses on aging processes during wine production. Aging is a dynamic process involving both volatile and nonvolatile compounds. During this process some compounds degrade, whereas other compounds form. Understanding these processes are of economic importance, particularly for wine since aging can be such a critical step in its production.

This symposium book is a unique volume that describes the advances in flavor chemistry research related to alcoholic beverages. It will be an excellent reference book for all scientists and professionals engaging in the research and development in the field of food and beverage flavoring and flavor ingredients. We are grateful to the authors for their contributions as well as to the reviewers for their valuable critiques.

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Editors' Biographies

Michael C. Qian

Michael C. Qian, Ph.D., is a faculty member at Oregon State University. He received his B.S. degree in Chemistry from Wuhan University of China, his M.S. degree in Food Science from the University of Illinois at Urbana-Champaign, and his Ph.D. from the University of Minnesota under the guidance of Dr. Gary Reineccius. Dr. Qian's research interests at Oregon State University focus on flavor chemistry and instrumental analysis involving solventless sample preparation such as solid phase micro-extraction, solid phase dynamic extraction, stir bar sorptive extraction, and fast GC, multi-dimensional GC/GC-MS analysis of volatile aroma compounds. His research area has covered aroma/flavor chemical/biochemical generation in dairy products, small fruits (blackberries, raspberry, and strawberry), wine and wine grapes, beer, and hops. He has made significant contributions to the field of flavor chemistry. He has published more than 50 peer-reviewed original research papers and 12 book chapters in the field of flavor chemistry and analytical chemistry. He has previously co-edited *Volatile Sulfur Compounds in Food*, *Flavor and Health Benefit of Small Fruits*, and *Micro/Nano-encapsultion of Active Food Components*, published by the American Chemical Society, and is a frequent speaker at national and international meetings. Before he came to academia, Dr. Qian spent 10 years in industry as a research scientist. Dr. Qian was a former chair of ACS AgFd Flavor Sub Division and is currently serving as vice chair of the Agricultural and Food Chemistry Division of ACS.

Thomas H. Shellhammer

Thomas H. Shellhammer, Ph.D., is the Nor'Wester Professor of Fermentation Science in the Department of Food Science and Technology at Oregon State University where he leads the brewing science education and research programs. His brewing research investigates hops and beer quality, hop-derived bitterness and its quality assessment, and the origins of hop aroma and flavor in beer. He directs the brewing education component of the Fermentation Science program at OSU and teaches courses about brewing science and technology, beer and raw materials analyses, as well as an overview of the history, business, and technology of the wine, beer, and spirits industries. Dr. Shellhammer received his Ph.D. from the University of California, Davis in 1996. During the 2008−2009 academic year, while on sabbatical leave from OSU, he worked at the Technical University of Berlin as a Fulbright Scholar and Alexander von Humboldt Fellow. Dr. Shellhammer is a member of the Board of Examiners for the Institute of Brewing and Distilling, London, England, a Fellow of the Institute of Food Technologists, and the Chairman of the Editorial Board of the MBAA *Technical Quarterly*.

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Chapter 1

Spice Up Your Life: Analysis of Key Aroma Compounds in Shiraz

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Shiraz is Australia's most important red grape variety, and is essential for producing a unique diversity of red wine styles, including some of Australia's 'icon' wines. Anecdotal evidence suggests that a spicy, 'pepper' aroma is important to some high quality Australian Shiraz wines. Despite the significance of Shiraz to the Australian wine sector, little is known about the aroma compounds that are the key contributors to the perceived aroma and flavour of premium quality Shiraz wine, and the compound responsible for this distinctive 'pepper' aroma in Shiraz had eluded identification until recently. In this paper we summarise the untargeted metabolomics approaches and GC-MS-O experiments employed for the identification of key Shiraz grape and wine sesquiterpenes, α-ylangene (Parker et al. *J. Agric. Food Chem.* **2007**, 55, 5948–5955) and rotundone (Wood et al. *J. Agric. Food Chem.* **2008**, 56, 3738–3744). The relatively unknown sesquiterpene rotundone was identified as an important aroma impact compound in grapes, wine, and common spices with a strong spicy, peppercorn aroma. An aroma detection threshold of 16 ng/L in red wine indicates that rotundone is a major contributor to peppery characters in Shiraz grapes and wine, and to a lesser extent in wine of other varieties, and we explore some factors that influence rotundone concentrations in wine.

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Introduction

Shiraz is one of the world's top six grape varieties along with Merlot, Cabernet Sauvignon, Pinot Noir, Sauvignon Blanc and Chardonnay. The vineyard area planted to Syrah/Shiraz vines has grown from less than 10,000 hectares in the early 1980s to more than 140,000 hectares in 2004/2005. About 50% of Shiraz is grown in France, and 25% in Australia, with Argentina, South Africa, California, Chile, USA, Italy, New Zealand, Greece, Spain, Switzerland and other smaller producing countries accounting for the remainder. Shiraz is Australia's favourite red wine variety, accounting for 51.4% of the total crush of red grapes or 25.8% of total wine grape production of 1.6 million tonnes in 2009/10 (*1*).

Shiraz (the name used by many New World producers for the grapevine variety known as Syrah in France) is an ancient variety and is thought to have emerged from Mondeuse blanche and Dureza in the northern Rhône Valley, ca. 100 AD (*2*); it was also one of the first vine varieties to arrive in Australia in 1832. To date, grapes are still used for winemaking from own rooted Shiraz vines that have been planted in Australia more than 120 to 160 years ago in the Hunter Valley, Victoria and the Barossa Valley. Shiraz wines have interesting and diverse aromas ranging from plum, berries and chocolate to liquorice and spice, depending on the regions. Shiraz is a very versatile variety and is used on its own or in blends with Cabernet Sauvignon, with Grenache and Mourvedre, or Viognier. Prominent Australian Shiraz styles include elegant, peppery cool-climate wines (for example from the Adelaide Hills, or the Grampians); more intensely flavoured, spicy and sometimes minty styles of Margaret River, Coonawarra or Clare Valley; sweet chocolaty, muscular and ripe-fruited wines (Barossa Valley, McLaren Vale), and leathery and rich wines (Hunter Valley). To illustrate the range of sensory attributes commonly found in Shiraz wine, Figure 1 compares the sensory profiles generated by a trained sensory descriptive panel of two wines from a cooler and a warmer grape-growing region (*3*). Clearly, the wine from the cooler Margaret River region (06MR) was rated significantly higher in 'pepper' aroma, 'astringency' and 'acidity'. In contrast the Shiraz from the Barossa Valley (06BV) had significantly more 'overall fruit', 'dark fruit', and 'jammy fruit' aroma and flavour.

Despite the importance of Shiraz to the Australian wine industry, little was known until recently about the aroma compounds that are the key contributors to the perceived aroma and flavour of premium quality Shiraz wine. Anecdotal evidence, tasting notes, and the backlabels of Australian Shiraz wine bottles suggested that a 'spicy', 'pepper' aroma is important to some high quality Australian Shiraz wines. The pepper character could be thought of as quintessentially Australian and possibly may even form part of the 'terroir' for a particular wine, yet the compound(s) responsible for this distinctive aroma in Shiraz had not been identified. Thus it was important to isolate and gain a greater understanding of such a powerful odorant that is present in grapes and wine in our own backyard.

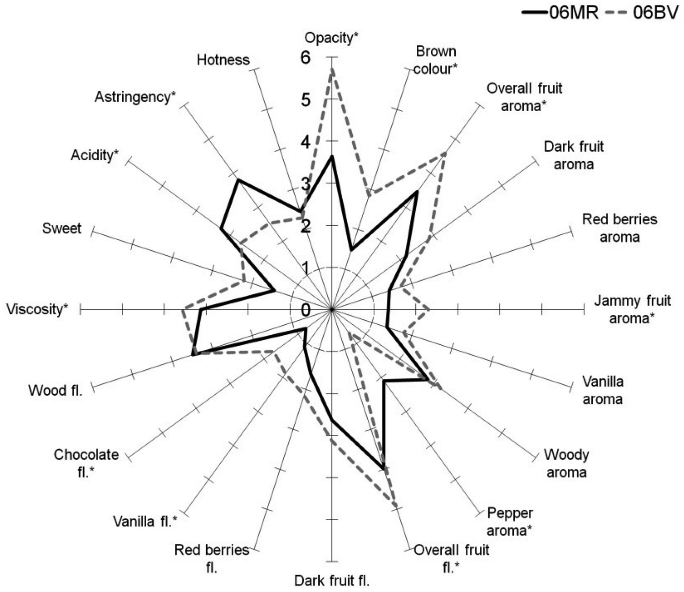

Figure 1. Radar plot of the mean sensory data for two ultra premium Shiraz wines from a cool (solid black line) and a warm (dashed grey line) grape-growing region. Asterisks indicate statistically significant attributes ($p < 0.05$). fl: flavour.

Materials and Methods

Experimental details about grape samples, sensory evaluation of 'peppery' aromas in grape homogenates, GC-MS analysis of sesquiterpenes and the untargeted GC-MS metabolomics strategy that led to the identification of the Shiraz grape sesquiterpene, α-ylangene, as marker for 'pepper' aroma have been described by Parker and co-workers (*4*). The GC-MS-O experiments and sensory studies to identify rotundone as important impact compound with a strong 'spicy', 'pepper' aroma have been summarised in (*5*), and the analytical method used to quantify rotundone has been described in (*6*) by Siebert and co-workers.

For the consumer sensory study (*7*), rotundone was added at two concentrations, at 25 ng/L and 125 ng/L, guaiacol was added at 25 and 50 µg/L, and eucalyptol was added at 4 and 30 µg/L to a relatively low flavour bag-in-box Merlot base wine that had no detectable level of rotundone (less than 5 ng/L), and had very low levels of guaiacol and eucalyptol (5 and 0.18 µg/L respectively). The six individually spiked wines plus the Merlot base wine were profiled by 10 trained AWRI panellists who evaluated the wines in triplicate. The same wines were assessed by 104 consumers in Adelaide who were recruited based on their red wine consumption of at least one glass per week. All samples were served blind in ISO tasting glasses for both consumer testing and trained panel sensory evaluation. Wines were identified only with a three-digit code and were served in a sequential monadic and randomised order to minimise any bias. Consumers

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rated each wine for overall liking on a nine point hedonic scale, together with purchase intent on a five point scale, followed by a number of questions to explore their attitudes towards wine.

Results and Discussion

In early experiments, many extracts of Shiraz grapes were investigated by gas chromatography with olfactory detection (GC-O) and gas chromatography-mass spectrometry (GC-MS), but no single region or known compound corresponding to a distinctive 'spicy' or 'pepper' aroma could be found (*8*). However, the 'black pepper' flavour could be perceived in individual berries and deseeded Shiraz grape berry homogenates. Based on anecdotal evidence that there are 'peppery' vineyards that consistently produce 'peppery' wines, especially in cooler years, a large sample set of potentially 'peppery' grapes was sourced from 12 vineyards in South Australia and Victoria. The important sensory attributes of 18 grape samples, including the aroma descriptor 'pepper', were rated by sensory descriptive analysis (*4*). This 'black pepper' attribute was independent of the 'green', 'grassy', and 'raisin' attributes also present. The sensory study revealed a strong correlation between the intensity of 'pepper' aroma and the intensity of 'pepper' flavour on the palate and enabled us to concentrate on grape volatiles for further experiments. Chemical analyses of these grape samples were carried out for pH, TA, and TSS. However, there were no significant trends relating any of these standard maturity and quality measures of the grapes to their sensory 'pepper' scores.

To study all grape volatile metabolites in a comprehensive, nontargeted fashion, grape homogenate samples were analyzed by static headspace GC-MS. For the metabolomics experiments a cool inlet system was used, we achieved enrichment of trace volatile aroma compounds for improved limits of detection in the low ppb-range, and avoided undesirable discrimination and matrix effects from sampling techniques such as SPME. This GC-MS analysis yielded over 13000 individual mass spectra per grape sample. Prior to multivariate data analysis the data were preprocessed using smoothing and mean normalisation procedures. To explain the intensity of the rating of the 'pepper' character, principal component analysis and partial least-squares regression were then used to develop multivariate models based on mass spectra and aroma descriptors. Optimisation of the methodology enabled selection of a single region of the GC-MS chromatogram that allowed prediction of 'pepper' aroma intensity with a correlation coefficient >0.98. This led to the identification of α-ylangene, a tricyclic sesquiterpene, which was confirmed through co-injection with an authentic reference compound. Although not a significant aroma compound by itself, α-ylangene was a very good marker for the 'pepper' aroma in grapes and wine, and its concentration showed similar discrimination between 'peppery' vineyards and vintages as that obtained using the multivariate models (*4*).

Notably, multivariate analysis of data from metabolomics experiments typically results in the identification of key features and metabolites based on correlation with other metadata, but does not establish cause-effect relationships.

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In this example, we were able to robustly identify a single sesquiterpene marker, α-ylangene, at trace concentrations of 1 to 15 μg/kg through an untargeted GC-MS experiment and in the presence of a range of other sesquiterpenes. At the same time we missed out on detecting the key aroma impact compound due to its very low odour threshold and concentration. This example shows that metabolomics strategies can complement established approaches to identify bioactives, impact aroma compounds and other labile trace compounds. The subsequent identification of rotundone, the 'peppery' key aroma impact compound in extracts from *Piper nigrum* and Shiraz berries, required traditional GC-MS-O experiments, and succeeded only after sensory-guided, elaborate optimisation of sample preparation and enrichment (*5*). It was further complicated by the unusal late elution time of rotundone towards the end of the GC-MS-O analysis. Finally, the presence of rotundone was confirmed in the enriched pepper and grape extracts by GC-MS-O and co-injections with increasing amounts of the synthesised compound, which gave symmetrical peak enhancement, a matching mass spectrum, and the distinctive pepper aroma only at the correct retention indices on three GC column phases (DB-5, DB-1701, and Wax).

Sensory Properties of Rotundone

Once the identification of the sesquiterpene rotundone as aroma compound had been verified with the help of a reference substance, we developed a method to robustly quantify rotundone by stable isotope dilution analysis (SIDA) and GC-MS (*6*), and conducted sensory experiments to better understand its aroma properties. Excellent correlations were observed between the concentration of rotundone and the mean 'black pepper' aroma intensity rated by sensory panels for both grape and wine samples, indicating that rotundone is a major contributor to peppery characters in Shiraz grapes and wine. Furthermore, sensory thresholds for rotundone were determined to be 8 ng/L in water and 16 ng/L in red wine (*5*).

Notably, approximately 20% of sensory panellists could not detect rotundone during the threshold testing even at 500 times the best estimate detection threshold in water (*5*). Thus, the sensory experiences of two consumers enjoying the same glass of Shiraz wine might be very different. To follow on from this observation, a sensory study assessed the effect of rotundone (black pepper), along with eucalyptol (mint, camphor, eucalyptus) and guaiacol (smoky) when added at moderate and high levels to a red wine. This study explored consumer preferences and tolerances to naturally occurring flavour components in wines normally described as peppery, eucalyptus and smoky to understand desirable levels of these compounds in wines. The sensory properties were determined by a sensory descriptive panel, and 104 Adelaide consumers tasted the wines and gave liking scores. Through the descriptive study it was demonstrated that the attributes 'red berry', 'dark berry', 'vanilla', 'smoky', 'pepper', 'mint/eucalyptus', 'vanilla palate', 'smoky palate', 'mint/eucalyptus palate', and 'pepper palate' were significantly different among the samples ($P<0.05$). From the liking scores three groups of consumers with similar preferences could be identified by cluster analysis, with roughly equal proportions of consumers in each group. Figure 2 shows the results of the consumer testing (*7*).

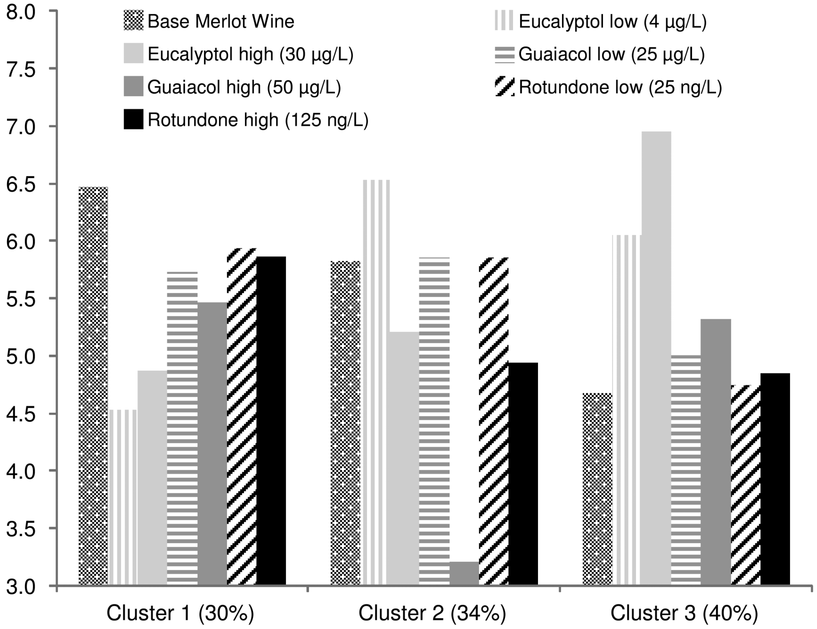

Figure 2. Consumer liking scores for a Merlot wine with added flavour compounds (7).

Consumers are not uniform in their preferences and different groups of consumers often respond differently to wine flavours. The consumers in Cluster 1 liked least the wines with added eucalyptol. Cluster 2 consumers disliked the high guaiacol wine, while Cluster 3 very much preferred the base wine with added eucalyptol. The addition of 25 ng/L rotundone had little effect on the consumers' preferences, and a dose effect for rotundone was only apparent for the consumers in Cluster 2 where the red wine with the higher concentration of rotundone (125 ng/L) was given a lower liking score. Overall, rotundone addition was positive for a third of the consumers and fairly neutral to the rest. Preferences and tolerances for the different flavours thus vary considerably among consumers with distinct niches of consumers preferring specific flavours. To assess the effects of rotundone on quality as perceived by consumers further work is required with other base wines and, for rotundone, in the presence of additional compounds that influence 'acidity', 'green', 'berry' and 'overall fruit' flavours.

Occurrence of Rotundone in Commercial Wine

With the identification and analytical method development hurdles overcome, we started testing some of the factors that may contribute to pepperiness, such as grape variety, cultivar, clone type and region. To assess the distribution of rotundone and to help guide further studies rotundone analyses were undertaken

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of a large range of commercially available Australian wines (137 predominantly red wines obtained from local retailers) of different varieties and vintages from various regions (*9*). The majority were bottled either under screwcap or natural cork and included Shiraz, Merlot, Durif, Pinot Noir, Cabernet Sauvignon and several other interesting wines from popular winegrowing regions from the early 1990s until 2006. Figure 3 shows the amounts of rotundone encountered and wine variety/region in samples where the compound was present. The vast majority (81%) of the wines had no detectable rotundone, and of the wines that contained rotundone, 62% were Shiraz. From Figure 3 it is also apparent that above-threshold levels of rotundone (>16 ng/L) are often encountered in wines originating from cool climate regions and/or colder vintages, and are not limited to Shiraz. This is in agreement with previous observations (*5*, *10*) and recent results obtained by analysis of Schioppettino, Vespolina and Grüner Veltliner wines produced in Europe (*11*, *12*). Beyond grapes and wine, rotundone was found in much higher amounts in other common herbs and spices, especially black and white peppercorns, where it was present at approximately 10000 times the level found in very 'peppery' wine (*5*).

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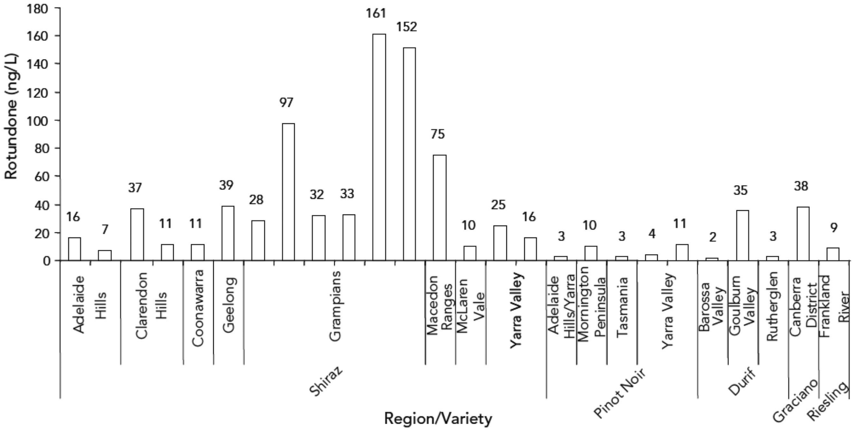

Figure 3. Rotundone concentration in commercial Australian wine (9).

To characterise the stability of rotundone in wine during ageing, an ongoing study is looking at the effects of several closures on rotundone levels in bottled wine (*9*). To determine whether the compound is 'scalped' by the closure, as is the case with other aroma compounds (*13*), Shiraz wine was spiked with rotundone at approximately 100 ng/L. Bottles (750 mL; 24 for each closure) were sealed with either natural cork, synthetic cork, or stelvin screw cap and sealed glass ampoules were prepared as controls at the time of bottling. Triplicate samples were analysed for rotundone after 0, 6, 12 and 39 months. There was no change in rotundone levels until 39 months, whereupon minimal scalping by the synthetic closure was observed (~6% reduction based on the original concentration). The stability of rotundone under wine-like conditions and the relative lack of scalping of the compound indicate that the pepper characteristics of a particular wine at bottling are unlikely to change drastically over time with proper storage conditions. Indeed, a Shiraz wine from the Grampians region with the highest level of rotundone (161 ng/L) appearing in Figure 3 was from the 2002 vintage, while another Grampians region Shiraz from 1999 still had 152 ng/L present some 10 years after bottling. These examples indicate the relative stability of the compound over many years (*9*).

Factors Influencing the Concentration of Rotundone in Grapes and Wine

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Rotundone is quite unusual for a wine aroma compound as it is one of a small group of important impact aromas (such as isobutyl-methoxypyrazine or some monoterpenes) that stem directly from grapes. We assume that rotundone present in a wine would have been extracted without any further chemical or biochemical transformation during winemaking. In contrast it is much more common that volatile wine aroma compounds are released from their odourless precursors (such as glycosides, or cysteine-*S*-conjugates) or that they are formed by the yeast entirely during fermentation. Based on the direct grape-to-wine relationship for rotundone (*5*, *12*), and given the low sensory threshold for rotundone (*5*) and its apparent stability in wine (*9*), this opens opportunities to influence the level of rotundone, and 'pepper' aroma and flavour in wine through clonal selection, appropriate viticultural practices or by varying winemaking procedures. But first we needed to find out when rotundone develops in the berries, where it is localised and how much is extracted from berries during winemaking.

As climate is known to impact on grape and wine rotundone concentrations (*5*, *9*), an Adelaide Hills vineyard, planted with Shiraz clones 1127 and 2626, was selected for this study because of its cool climate and regular production of moderately 'peppery' Shiraz grapes. All samples were analysed as per the previously published method (*6*). To monitor rotundone levels in the berries during ripening, bunch samples were taken from comparable rows of both Shiraz clones at veraison, 50% red colouring midway between veraison and harvest; and one day before commercial harvest. At early ripening stages we measured only low levels of rotundone in the berries (typically below 5 pg/berry) until well after veraison, with most of the rotundone accumulating in the last six weeks of ripening. At harvest, a higher rotundone concentration of 20 pg/berry was found in Shiraz clone 2626, which is in agreement with the anecdotal belief that 2626 is a 'spicier' Shiraz clone (*14*).

To investigate the location of rotundone in Shiraz grapes, we analysed fresh harvest samples, skins separated from pulp, juice and seeds, and pulp and juice with seeds removed. Rotundone was only found in the skin of the Shiraz berries and not detected in the pulp, juice or seeds after separation. While this study involved only a limited sample set, and more work is required before general conclusions can be drawn, the finding that rotundone is located in berry skins is consistent with other research (*8*, *12*). In the skins of Shiraz clone 1127, rotundone was quantified at 24.7 ng/kg, and at 49.5 ng/kg in clone 2626. Again, clone type appeared to play a role, with a higher level of rotundone found in the Shiraz 2626 clone (*14*).

The extraction of rotundone from the berries into the wine was explored by measuring the concentration of rotundone in samples taken daily during the commercial fermentation of the two clones, from the initial must to the pressed wine. Grapes were commercially picked on the same day and at similar ripeness except that grapes from Shiraz clone 1127 were harvested and fermented in one tank and grapes from clone 2626 were split into three separate batches. The winemaking parameters were the same for all ferments apart from the day of pressing. As shown in Figure 4, most of the rotundone was extracted from the berries between days 2 and 5, and rotundone concentrations reached a plateau in all fermentations prior to pressing. Overall, the data are consistent with extraction of rotundone from the skins during fermentation, and the lag phase between crushing at day 0 and day 2 (day 3 for the fermentation of grapes from clone 1127) indicates that ethanol concentration and/or other yeast-related effects are likely involved in facilitating extraction of rotundone. In this preliminary study no significant difference in the concentration of rotundone was found between the two clones, with rotundone in the wines ranging from 30 to 38 ng/L. As the ferments utilised large batches of grapes, the observed differences are indicative of some variability of rotundone concentration in grapes across the vineyard, rather than demonstrating clonal effects (*14*).

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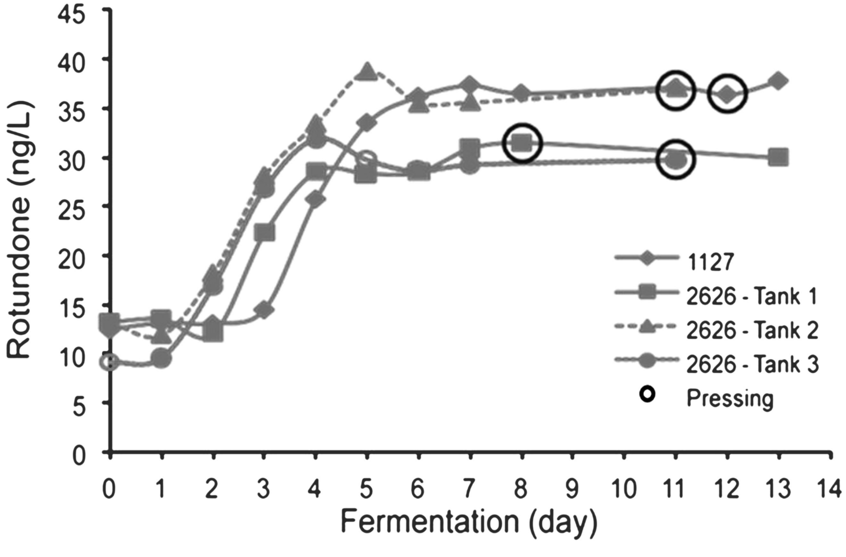

Figure 4. Rotundone extraction from berries during winemaking of Adelaide Hills Shiraz in 2009 (14).

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Conclusions

Rotundone, an oxygenated sesquiterpene, is the potent aroma compound responsible for ‘pepper’ aroma in grapes and wine. Rotundone is quite unusual for a wine aroma compound as it stems directly from grapes, has a very low sensory threshold, and is relatively stable in wine. This opens opportunities to influence the level of rotundone in wine, ‘pepper’ aroma and flavour, and wine style and consumer preferences through clonal selection, appropriate viticultural practices or by varying winemaking procedures.

While clonal effects may play some role for influencing rotundone concentration in Shiraz grapes, the data obtained so far indicate that rotundone biosynthesis is likely to be associated with an interaction of the grapevine genome with its environment: This hypothesis is based on the propensity of rotundone to be predominantly present in the variety Shiraz, with significantly elevated concentrations typically observed in some vintages, and for grapes grown in cool-climate vineyards. Also, in other plant species it has been demonstrated that induction of sesquiterpene biosynthesis is a common plant response to environmental pressures (*15*). Obviously, there is much scope for more detailed research to help researchers, grapegrowers and winemakers to understand how we can manage rotundone biosynthesis and concentration in grapes and may take advantage of its sensory effects in wine.

Acknowledgments

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Chapter 2

Analytical Investigations of Wine Odorant 3-Mercaptohexan-1-ol and Its Precursors

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We have developed and applied methods for the analysis of wine odorant 3-mercaptohexan-1-ol (3-MH) and its precursors (including the newly identified cysteinylglycine conjugate) in grape juice and wine. Studies which assessed the effects of grape ripening and processing operations highlighted some important findings. We identified the presence of 3-MH in unfermented juice for the first time and found a dramatic increase in precursor concentrations in the later stages of ripening. We also revealed the effects on precursors from freezing, transportation, fining and inhibiting grape enzymes. Additionally, using labeled (*E*)-2-hexenal we propose the role of the glutathione-aldehyde adduct as the first intermediate in the formation of 3-MH.

Introduction

Among the important grape-derived odorants contained in wine, one group of compounds – polyfunctional thiols – is predominantly associated with Sauvignon Blanc varietal character. The aromas of these "varietal" thiols have been described as "box tree", "tropical" and "passion fruit" and they are important contributors to wine quality (*1*). The key thiols for Sauvignon Blanc wine aroma, 4-mercapto-4-methylpentan-2-one (4-MMP), 3-mercaptohexan-1-ol (3-MH)

In Flavor Chemistry of Wine and Other Alcoholic Beverages; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

and 3-mercaptohexyl acetate (3-MHA), have extremely low aroma detection thresholds (Table I). The corresponding odor activity values (OAV) of these thiols, used as a measure of their sensory significance, can number in the hundreds. In particular, 3-MH and 3-MHA have frequently been found in concentrations well above their aroma detection thresholds in Sauvignon Blanc wines (*2*), especially those from France (*3*) and New Zealand (NZ) (*4*). As a result of their abundance and powerful aromas, varietal thiols in wine can influence consumer perception, affecting the level of preference for a particular wine (*1*, *4*).

Table I. Characteristics of varietal thiols found in Sauvignon Blanc wine

	Aroma detection threshold	*Aroma description*	*Concentration found in wine*	*Odor activity value*	*References*
4-MMP	3 ng/L	Blackcurrant Box tree Passionfruit	Low ng/L	Up to 30	(*2*, *3*, *5*)
3-MH	60 ng/L	Grapefruit Passionfruit	Low ng/L to low µg/L	Up to 210 (310 for NZ wine)	(*3*, *4*, *6*)
3-MHA	4 ng/L	Passionfruit Box tree Sweaty	Low ng/L to low µg/L	Up to 195 (625 for NZ wine)	(*3*, *4*, *7*)

Since 3-MHA arises from 3-MH during fermentation (*8*), we focused on factors associated with 3-MH formation. Although often treated as one compound, 3-MH is present in wine as a mixture of enantiomers (Figure 1), each with different aroma detection thresholds and descriptors. (*R*)-3-MH has an aroma described as "grapefruit" with a threshold of 50 ng/L whereas (*S*)-3-MH has an aroma described as "passionfruit" and a threshold of 60 ng/L (*9*). Given their impact, it is essential to understand how these compounds are formed and factors that relate to their stability in wine in order to optimize wine sensory characters as desired.

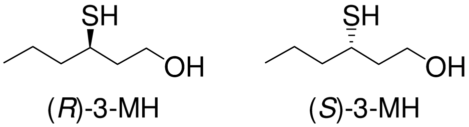

Figure 1. Structures of the 3-MH enantiomers found in wine.

Origins of 3-MH in Wine

3-MH can be generated from odorless precursors which are present in grape juice. The free thiol has not been found in unfermented juice in high concentrations as thiols are released by carbon-sulfur lyase (CSL) activity during vinification (*10–14*). 3-MH can be further modified by yeast acetyl transferase (ATF) enzymes to generate 3-MHA (*8*).

Precursors to varietal thiol 3-MH, derived from cysteine (Cys-3-MH) (*12*) and glutathione (Glut-3-MH) (*15*) have been identified in Sauvignon Blanc juice. These precursors are present as pairs of diastereomers which each release the (*R*)- and (*S*)-3-MH enantiomers. More recently, the cysteinylglycine conjugate of 3-MH (Cysgly-3-MH), an intermediate precursor in the degradation of Glut-3-MH to Cys-3-MH, was identified in Sauvignon Blanc juices (*16*). As expected, based on its relationship to both Cys- and Glut-3-MH, this compound also exists as two diastereomers (Figure 2).

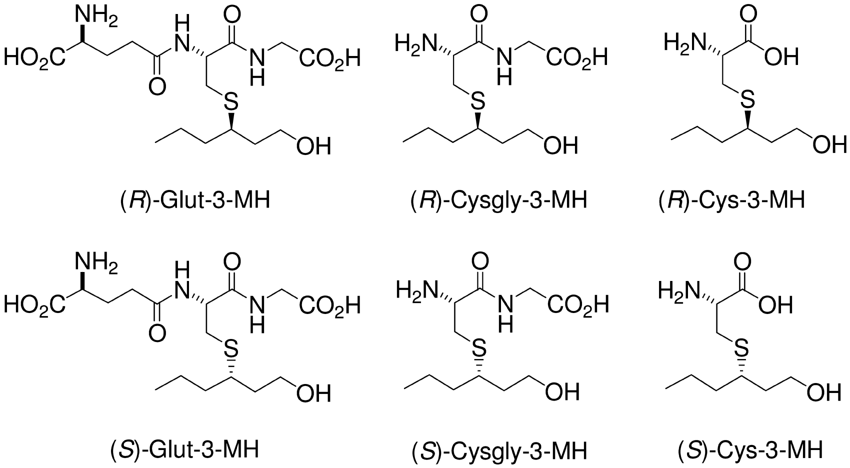

Figure 2. Structures of the diastereomers of Glut-, Cysgly- and Cys-3-MH found in grape juice. The stereochemical designations relate to the alkyl chain stereocenter.

A range of previous studies of precursors to 3-MH were limited to the cysteine conjugate (*3*, *11*, *17–19*) so we further probed the relationships between various 3-MH precursors in juice and 3-MH in wine. We investigated model fermentations of Cys- and Glut-3-MH with VIN13 and modified VIN13 yeast strains, revealing for the first time that yeast can also utilize the glutathione conjugate, leading to the formation of 3-MH (*20*). This work demonstrated that fermentation of pure (*R*)-Glut-3-MH resulted in an approximate 3% conversion to (*R*)-3-MH as a single enantiomer (Figure 3). (*R*)-Cys-3-MH was also formed during the transformation, presumably through the dipeptide intermediate

(*R*)-Cysgly-3-MH, but this remained to be confirmed. It appeared that Cys-3-MH was more easily transformed during fermentation compared to its Glut-3-MH counterpart, since the conversion yield of 3-MH from Cys-3-MH was in the order of 14% (*20*). This observation has since been supported by other studies (*21–23*).

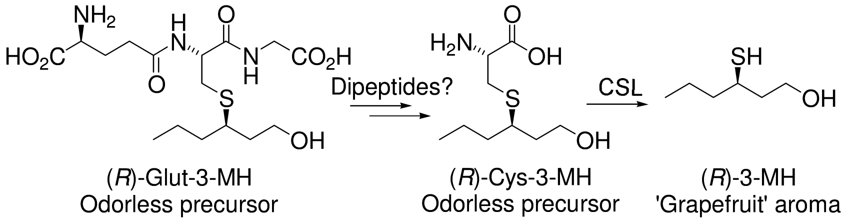

Figure 3. Fermentation of a single diastereomer of Glut-3-MH ultimately leading to one enantiomer of 3-MH. Other intermediates could include the dipeptide Cysgly-3-MH. The (R)-designation relates to the alkyl chain stereocenter.

Determination of 3-MH Precursors in Juices and Wines

Methods for the quantitation of 3-MH precursors in musts or wines had been confined to assessment of the cysteine conjugate (*24*), most often without resolving the diastereomers. Several methods have utilized GC-MS analysis of Cys-3-MH either indirectly (*24*) or after derivatization (*17*, *18*, *25*) while an HPLC-MS method has also been reported for determination of the unresolved Cys-3-MH diastereomers (*26*).

We recently developed a stable isotope dilution analysis (SIDA) method for 3-MH precursors in juices and wines which resolved both diastereomers of Cys- and Glut-3-MH using HPLC-MS/MS (*27*) and subsequently added Cysgly-3-MH to the method (*16*). This was the first method where the individual diastereomers of Cys-, Cysgly- and Glut-3-MH were determined in a single analysis. Resolution of diastereomers will be important when studying the evolution of 3-MH enantiomers during winemaking and storage. Cysteine and glutathione conjugates of 3-MH have also been analyzed by Roland et al. (*28*) using a nanoLC-MS/MS SIDA method (included conjugates of 4-MMP), Kobayashi et al. (*21*) using HPLC-MS/MS without internal standard and Allen et al. (*29*) using SIDA and a modified procedure based on that of Capone et al (*27*). None of these methods resolved the diastereomers of the 3-MH conjugates.

Some concentration ranges for Cys- and Glut-3-MH previously found in juice and wine appear in Table II. While there was good accord with Cys-3-MH concentrations in juice, Glut-3-MH varied considerably between the two reports; this could be due to differences in sample origin or preparation as described below. Analysis of a range of wine samples showed that significant quantities of precursors remained in wine (Table II). This might affect in-mouth release and retronasal perception of 3-MH upon wine consumption (*30*) or lead to liberation of free thiols during storage. Furthermore, we found that Pinot Gris, Chardonnay and Riesling juices contained appreciable quantities of 3-MH precursors, but generally Sauvignon Blanc juices were highest (*27*). Roland et al assessed Melon B., Riesling, and Gewurztraminer juices as well as Sauvignon Blanc, and found that Gewurztraminer typically had the greatest amounts of 3-MH precursors, while Melon B. had the least (*28*).

Table II. Concentrations of 3-MH precursors determined for commercial Sauvignon Blanc juices

	Capone et al 2010 Juice (27)[a]	*Roland et al 2010 Juice (28)*	*Capone et al 2010 Wine (27)* [a]
Cys-3-MH	21 – 55 µg/L	8 – 40 µg/L	1 – 35 µg/L
Glut-3-MH	245 – 696 µg/L	1 – 8 µg/L	138 – 142 µg/L

[a] Sum of individual diastereomers for each precursor type.

Determination of 3-MH in Wines

Due to their extremely low concentrations and reactivity, thiol compounds are difficult to measure at near-threshold levels in wine. A common method for extracting these compounds employs *p*-hydroxymercuribenzoate (*p*-HMB) solutions to selectively bind the thiols, followed by ion exchange chromatography (*5*). Although potential problems exist (*p*-HMB solutions are highly toxic and the methods involve complex extractions), different versions of the *p*-HMB extraction method have been proposed (*31*, *32*). Other methods have employed derivatizing agents such as 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr), with on-fiber (SPME) or in-cartridge (SPE) derivatization (*2*, *33*, *34*). However, routine adoption of these methods has not been forthcoming for various reasons, including problems with linearity, repeatability and sensitivity (*34*, *35*). The methodology involving in-cartridge derivatization with PFBBr followed by SPME has again been improved upon (*2*, *35*), but as with previous methods, the approach requires negative chemical ionisation (NCI) mass spectrometry for sensitivity. GC-MS instruments with NCI capability may not be available in many laboratories and an electron ionization-mass spectrometry (EI-MS) method was considered to be a useful option.

Development of a Quantitative 3-MH Method for Application to Juices and Wines

We developed a modified SIDA method for analysing 3-MH in juices and wines for implementation in laboratories containing a GC with conventional EI-MS, and eliminated the need for extraction with mercury complexes (*36*). By combining liquid-liquid extraction and PFBBr derivatization, followed by SPME sampling of the headspace, we achieved excellent method precision (<2.5% RSD for a 25 ng/L spike into a wine containing 376 ng/L of 3-MH) and sub-threshold limits of detection and quantitation (30 ng/L and 40 ng/L, respectively).

Ripening and Fermentation

This new method has been applied in a number of studies, including an assessment of 3-MH evolution during ripening of Sauvignon Blanc clones (*36*). This was the first time that natural 3-MH was measured during grape ripening, although it has been incorrectly described as involving exogenous enzymatic treatment (*37*). 3-MH was barely detectable at veraison and increased to approximately 100 ng/L at mid-ripening, before remaining relatively static until harvest (Figure 4). Such concentrations were above the aroma detection threshold of 3-MH and the tropical fruit characters associated with varietal thiols were clearly evident around the mid-ripening time point when tasting these berries in the vineyard.

At harvest the grapes were crushed and fermented on a 20 L scale, using a single yeast strain (Maurivin PDM). Analysis of 3-MH after fermentation revealed that concentrations had increased as expected (Figure 4), although not to the levels found in many commercial Sauvignon Blanc wines we examined. The apparent reason for the modest 3-MH levels related to the fact that the samples were hand-harvested, whereas commercial operations often involve machine-harvesting. Some of our work has shown a major effect of processing on precursor concentrations (*38*) while Allen et al. (*29*) have reported an increase in wine 3-MH concentrations due to machine-harvesting. There may also be differences between research and commercial winemaking attributable to the scale of the operations, as indicated by results detailed elsewhere (*38*).

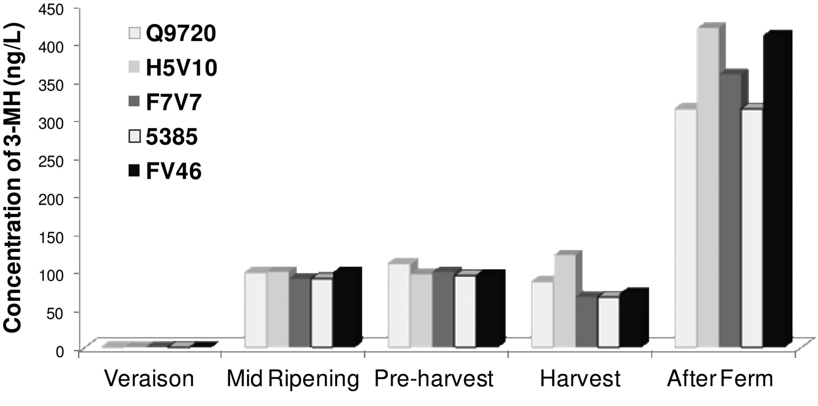

Figure 4. Concentrations of 3-MH (ng/L) determined during ripening of five Sauvignon Blanc clones. Ferm: Fermentation.

Analysis of 3-MH and Precursors

A survey of commercial white wine varieties was also undertaken, using our 3-MH and precursor SIDA methods (*36*). Figure 5 displays the 3-MH concentrations in a range of varietal wines, including Sauvignon Blanc, Muscat, Riesling, Pinot Gris and Gewurztraminer. While one Sauvignon Blanc in particular contained a high amount of 3-MH (3200 ng/L), other varietals also revealed significant quantities (around 1000 ng/L or more), indicating that 3-MH may be an important aroma compound in a range of grape varieties, especially when present well above its aroma threshold (*2*). In relation to the precursor diastereomer profiles for these wines, Glut-3-MH (up to 1240 nmol/L combined total) dominated over Cys-3-MH (up to 480 nmol/L combined total), the (*S*)-diastereomers were more abundant than the (*R*)-diastereomers, and Sauvignon Blanc generally contained the highest precursor concentrations (data not shown). These trends were consistent with our previous study on 3-MH precursors (*27*). Additionally, while high precursor concentrations are typically associated with Sauvignon Blanc, other varieties such as Muscats, Gewurztraminer and Riesling also contained appreciable quantities of precursors (data not shown), further confirming that the potential impact of 3-MH does not appear to be limited to Sauvignon Blanc wines.

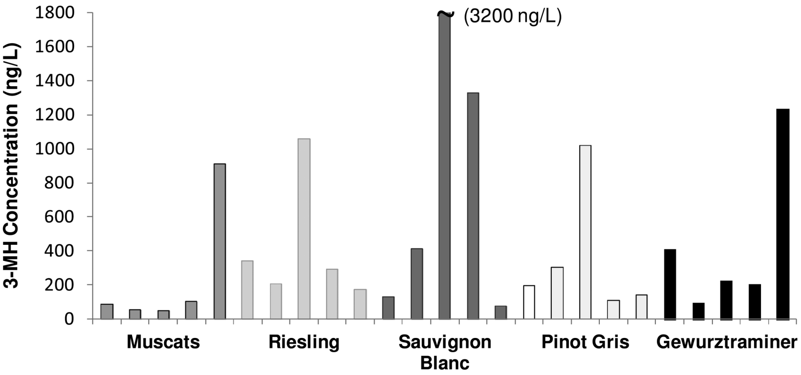

Figure 5. Concentrations of 3-MH (ng/L) determined in a survey of commercial Australian white wines.

Effects of Ripening on Precursor Concentrations

In a further investigation we evaluated precursor concentrations (Cys-, Cysgly- and Glut-3-MH) during ripening of Sauvignon Blanc, from veraison to several weeks past commercial ripeness (Figure 6, Cysgly-3-MH data not shown). This study was conducted using fruit from a University of Adelaide vineyard with bunch sampling every few days. Grapes were left on the vine longer than the usual practice to expand on our previous work, which showed a dramatic increase in 3-MH precursor concentrations in the lead up to commercial harvest (*36*). For the 2011 vintage, with the exception of Cysgly-3-MH which was barely detectable at any stage of ripening, 3-MH precursor concentrations generally increased during the ripening period up to a sugar level of about 24 °Brix (around ordinary commercial maturity), and then declined beyond this time. The increase in the lead up to harvest was consistent with our other results (*36*) and can be explained in terms of loss of membrane integrity (*39*, *40*) combined with an increase in available precursor constituents (*41*, *42*) with ripening. Sugar accumulation seemed to coincide with a decline in precursors (Figure 6) but the cause of the fluctuations in precursor concentrations was not clear, and may have been due to other metabolic changes caused by rainfall. Ultimately, factors relating to sugar accumulation, berry damage, rainfall and disease need to be considered, but the results reinforce the need for optimal harvest timing if precursor concentrations are to be maximized.

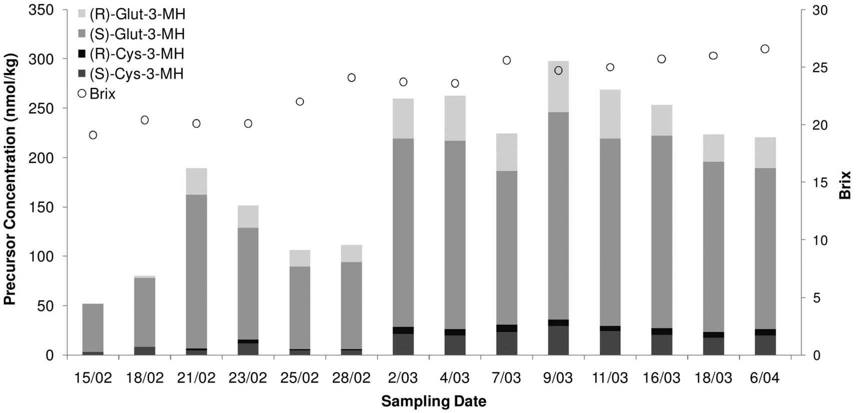

Figure 6. The effect of ripening on 3-MH precursors (nmol/kg) in Sauvignon Blanc fruit from a University of Adelaide vineyard during the 2011 vintage. Sampling occurred every 2-3 days until the last three time points. Rainfall: 29 mm on 19/2; 28 mm on 8/3; 13 mm on 9/3. The stacked bars represent the mean of each precursor diastereomer derived from three replicate samples. The relative standard deviations of the means were typically <20%.

Studies Directed toward Understanding the Effects of Processing on 3-MH Precursor Concentrations

Freezing

There are a number of reasons why grape or juice samples may need to be frozen prior to analysis so we assessed the impact of freezing grape bunches and grape juice on precursor concentrations (*36*). Fresh Sauvignon Blanc juices were prepared and analyzed, while portions were stored frozen, along with the corresponding grape bunches. After two months of frozen storage the samples were thawed and analyzed, with the thawed bunches being processed into juice consistent with the fresh juice samples. Freezing juice had little or no effect on precursor concentrations whereas freezing grapes had a dramatic effect for Glut-3-MH (Figure 7). While the fresh juice samples provided the usual concentrations of precursors (i.e. up to 270 nmol/L of Cys-3-MH and 880 nmol/L of Glut-3-MH), the Glut-3-MH concentrations after freezing (2000-4000 nmol/L) were the highest we have encountered in any of our studies. We attributed such increases to berry damage and formation of Glut-3-MH, rather than additional extraction due to freezing berries, since Cys-3-MH levels were barely affected. These observations, in conjunction with those from our berry ripening studies, suggested that Cys-3-MH was already present in the grape berry, while Glut-3-MH was formed upon berry damage. This was a strong indicator of the potential to influence Glut-3-MH concentrations as a result of processing options.

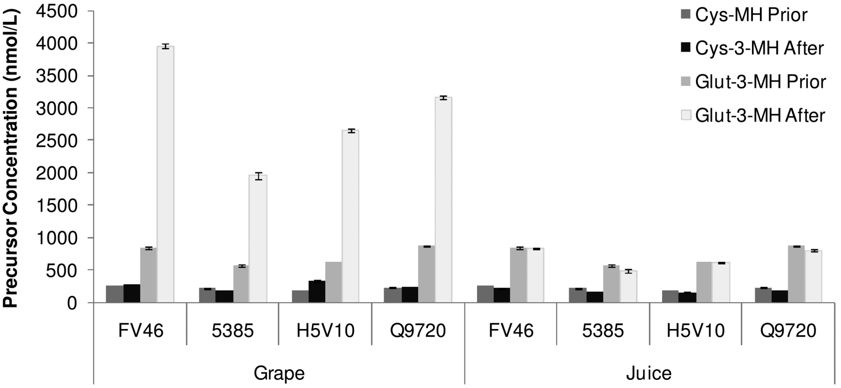

Figure 7. Mean 3-MH precursor concentrations (nmol/L) prior to freezing (Prior) and after freezing (After) for Sauvignon Blanc clone samples. Error bars represent the standard deviation of 3 replicate samples. Where the error bars are not visible the standard deviation was close to zero.

Fining

Fining of phenolics at the juice stage has the potential to avoid loss of aroma compounds that are formed only during or after fermentation. Therefore we measured Cys- and Glut-3-MH in Riesling free run, light and heavy pressed juices and heavy pressings which were fined using Liquifine (a gelatin fining agent) at 200 ppm (standard commercial rate of addition) and 1000 ppm (high rate of addition). The results in Figure 8 show that any merits of juice fining could also apply for preserving thiol precursors, since fining juice at the chosen rates had a negligible impact on precursor concentrations, whereas fining wine might to lead to some loss of varietal thiols. Comparing free run and pressed samples, there were higher concentrations of precursors with increased pressing, which was anticipated based on the effects of pressing shown by others (*43–45*). Although pressings may be used in lower grade products due to the additional phenolics present, substantial quantities of thiol precursors can potentially remain. If heavy fining of pressings can eliminate the harshest phenolics, then such pressings may actually have greater value due to their aromatic potential than previously recognized.

Transportation of Grapes

Our studies indicated that loss of berry integrity had a pronounced influence on Glut-3-MH concentrations. There was also anecdotal evidence from winemakers that increased tropical aromas were associated with wines made from transported fruit, and we gathered that substantial maceration must occur during transportation. Considering that fruit transportation is commonplace when vineyards and wineries are not situated in close proximity, we set out to assess the effects of this practice on thiol precursor concentrations. The work was carried out with the assistance of a large Australian winery so we could undertake experiments on a commercial scale with machine-harvested Sauvignon Blanc grapes (*38*).

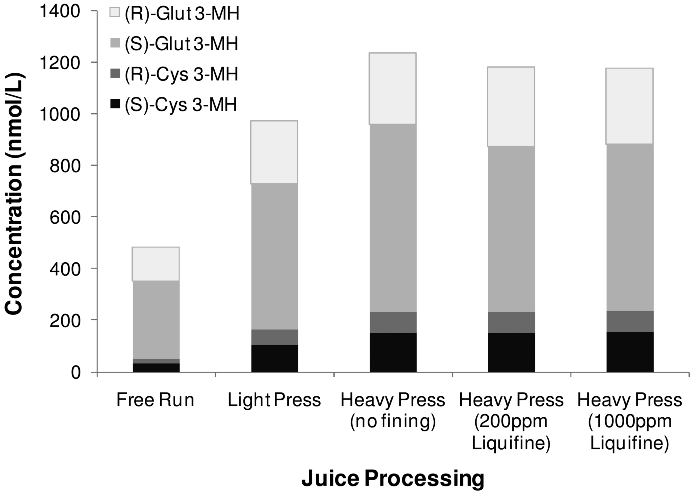

Figure 8. The impact of pressing and gelatin fining on 3-MH precursor levels (nmol/L) in Riesling juice. Juice volumes from 2 tonnes of grapes pressed sequentially were: Free run, 200 L; Light press, 200 L; Heavy press, 1000 L. The stacked bars represent the mean of each precursor diastereomer derived from three replicate samples. The relative standard deviations of the means were <15%.

Since antioxidants are typically used during winemaking to prevent oxidation, we also assessed the effect of antioxidants added at the time of harvest, using standard and very high rates of SO_2 and/or ascorbic acid addition. Triplicate 2.5 tonne bins had antioxidant treatments applied in the vineyard as outlined in Figure 9. Samples were removed from each bin in the vineyard and prepared in the laboratory for precursor analysis, while additional samples were obtained and prepared for analysis after transportation of the fruit by road (800 km in approximately 12 h).

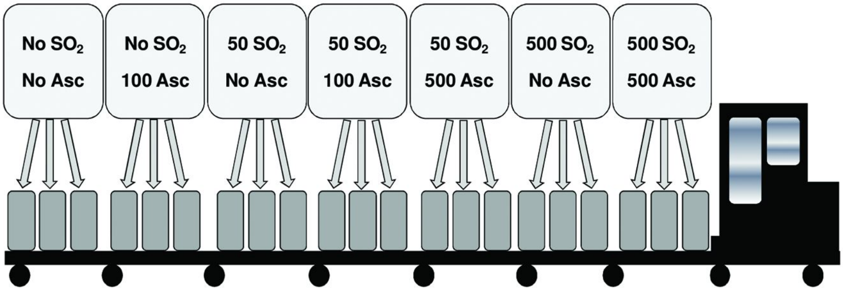

Figure 9. Antioxidant treatments applied at the vineyard (in mg/L) to machine-harvested Sauvignon Blanc fruit. SO_2 was added as potassium metabisulfite; Asc = ascorbic acid.

The results shown in Figure 10 highlight the large differences between the sample sets as a result of transportation. While the machine-harvested fruit contained expected Cys-3-MH concentrations (maximum of 180 nmol/L), there was up to a 10-fold increase as a result of fruit transportation, depending on the antioxidant considered (maximum of 1220 nmol/L). It is noteworthy that the samples with the lowest Cys-3-MH concentrations were those with the highest level of SO_2, while ascorbic acid had minimal influence. Cys-3-MH necessarily derives from Glut-3-MH, so the lower levels of Cys-3-MH in the presence of 500 mg/L SO_2 were likely an effect on Glut-3-MH formation or degradation. For the most part, the levels determined for Cys-3-MH in the transported fruit were substantially greater than those obtained in previous studies of healthy fruit by us (*36*) or others (*18*, *25*, *26*, *44*). Such unusually high concentrations likely arose from degradation of Glut-3-MH into Cys-3-MH, via Cysgly-3-MH as a result of grape berry or microflora enzymes. While the fruit we used was not visibly infected or damaged prior to harvest, our results were reminiscent of those reported by Thibon et al. for botrytized fruit, and their description of the enzymes involved was also pertinent (*46*).

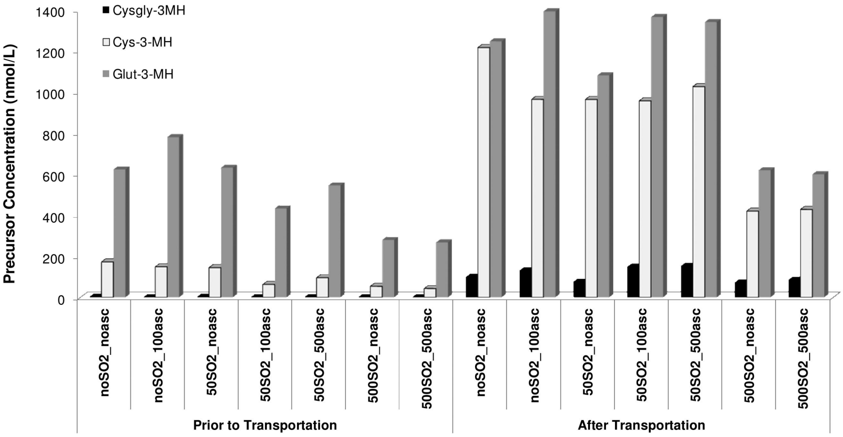

Figure 10. Mean concentrations of Cys-, Cysgly- and Glut-3-MH (nmol/L) before and after transportation in the presence of antioxidants. The bars represent the mean total of each precursor diastereomer derived from three replicate samples. The relative standard deviations of the means were typically <15%.

Glut-3-MH behaved similarly to Cys-3-MH, although in this case transportation did not yield as large an increase in glutathione precursor concentrations (Figure 10). Machine-harvesting afforded fairly modest Glut-3-MH concentrations (maximum of 740 nmol/L) while transportation led to an approximate doubling in concentration for most antioxidant treatments (maximum of 1400 nmol/L). Linked to the observations for Cys-3-MH, high levels of SO_2 afforded the lowest amounts of Glut-3-MH while ascorbic acid had virtually no effect. The role of SO_2 could be several-fold and may relate to inhibition of enzymatic transferase reactions or binding of (*E*)-2-hexenal, thereby preventing Glut-3-MH formation and/or degradation (*38*). The results reinforced the notion that additional Glut-3-MH could be formed post-harvest, but it was surprising to find it could be converted to sizeable quantities of Cys-3-MH given enough time. Indeed, this case of formation and degradation of Glut-3-MH, either partially or totally under the control of various enzymes, highlights the dynamic nature of the precursors such that processing methodology can play an important role. This is especially relevant because studies indicate that Cys-3-MH is more easily converted to 3-MH during vinification (*20–23*), so any process that can enhance its formation from Glut-3-MH could be very useful.

Cysgly-3-MH was only found in measurable quantities (maximum of 100 nmol/L) in fruit that had been transported (*16*), and high SO_2 had minimal impact on its concentration compared to the other precursors. Nonetheless, the presence of Cysgly-3-MH was of great interest to us, since this conjugate has only been found in trace concentrations in our ripening samples. It seemed as if Cysgly-3-MH was quickly transformed and the amounts in the transported fruit appeared to be some form of base level that had time to accumulate during the enzymatic transformation of Glut- to Cys-3-MH.

The implications of harvesting method were also examined by picking fruit by hand from the vineyard the day before it was harvested commercially. Analysis of these samples revealed precursor levels that were approximately 65% lower for Cys-3-MH and 70% lower for Glut-3-MH compared to machine-harvested fruit. While this indicated yet another factor that could influence precursor concentrations, the results contrasted with those of Allen et al. (*29*) who tended to find variable effects on precursor concentrations as a result of hand- and machine-harvesting. Nonetheless, a lack of mechanical berry damage could be an underlying cause for the low precursor levels encountered when fruit is hand-harvested.

In summary, for any given level of SO_2, different rates of ascorbic acid addition had minimal influence on precursor concentrations. In contrast, when SO_2 was used at an abnormally high level there was a large suppression of Glut-3-MH formation. Since it may be desirable to use some SO_2 in order to minimize oxidation of juices, as long as winemakers stay within the normal rates of application the effects on precursors should be relatively minor. A much more important effect arose as a result of fruit transportation, where a substantial amount of Glut-3-MH could be converted to Cys-3-MH. In fact, the final Cys-3-MH concentrations were roughly equivalent to the combined concentrations of both precursor types in the samples prior to transportation. Considering minimal Cysgly-3-MH was encountered, it would appear that it is a short-lived intermediate in the transformation of Glut- to Cys-3-MH that barely accumulates, even under conditions where Cys-3-MH is actively forming.

Inhibition of Grape Enzymes

Grape enzymes seem to play a critical role in precursor formation and Glut-3-MH concentrations may increase during post-harvest operations as a result of enzyme activity. Replicated experiments were therefore conducted to examine how much Glut-3-MH was present in grape berries compared to how much could be formed upon berry crushing. This was accomplished by snap-freezing fresh berries in liquid nitrogen, grinding them to a fine powder and using methanol/chloroform to precipitate proteins and minimize enzyme activity (*38*). It was of great importance to find that compared to ordinarily prepared juices, the Glut-3-MH concentration was reduced by up to 85% in the samples from enzyme inhibition, while Cys-3-MH concentrations were not so affected (data not shown). This was an additional piece of evidence to support the formation of Glut-3-MH as a result of loss of berry integrity, and it helped to explain the low Glut-3-MH results shown earlier by Roland et al. (*47*). If enzymatic or oxidative processes are prevented from occurring upon crushing of the berries then Glut-3-MH concentrations can be lower than that found from commercial processing. The consistent theme from the results of our studies led us to hypothesize that Cys-3-MH, arising via Cysgly-3-MH from the breakdown of Glut-3-MH, is endogenous to the berry and relatively impervious to short-term grape processing effects (in contrast to longer term processes such as fruit transportation). On the other hand, the largest amount of Glut-3-MH can be formed post-harvest and processing conditions can greatly influence juice concentrations.

3-S-Glutathionylhexanal (Glut-3-MHAl) as a New Intermediate in the Formation of Glut-3-MH

One puzzling aspect relating to Glut-3-MH was the general lack of acknowledgement in the literature of an aldehyde intermediate, given that formation has been assumed to involve conjugation of GSH with (*E*)-2-hexenal. We addressed this by undertaking replicated experiments to simultaneously verify a route to formation of Glut-3-MH and investigate the missing intermediate between conjugation of glutathione to (*E*)-2-hexenal in the process. To this end we added d_8-(*E*)-2-hexenal to whole Sauvignon Blanc berries prior to crushing them in a benchtop sample press. The juice was analyzed by HPLC-MS/MS in enhanced product ion (EPI) mode to monitor for products from the incorporation of labeled (*E*)-2-hexenal (*38*). The enhanced product ion spectra obtained for two compounds which showed incorporation of the d_8-labeled aldehyde can be seen in Figure 11. The presence of d_8-Glut-3-MH (*m*/*z* 416, Figure 11A) was easily confirmed based on a comparison of its fragmentation pattern with that of the known d_{10}-analogue (*27*). We could also identify d_8-Glut-3-MHAl (*m*/*z* 414, Figure 11B) based on it being 2 mass units less but with very similar fragmentation pattern to d_8-Glut-3-MH. Both spectra showed virtually identical neutral losses of fragments, ending ultimately with either the neutral loss of d_8-hexenol from d_8-Glut-3-MH or d_8-hexenal from d_8-Glut-3-MHAl. There was no detection of d_8-Cys-3-MH (m/z 230) in this experiment.

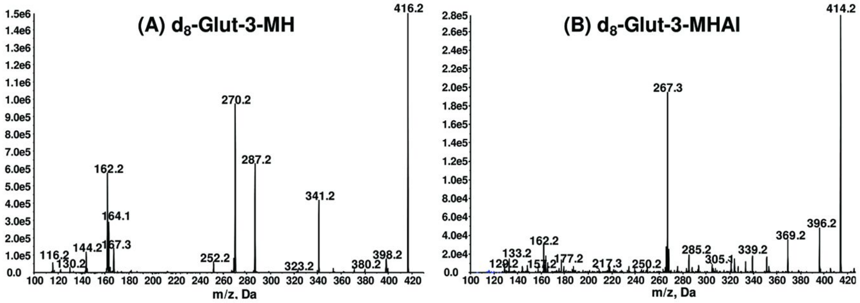

Figure 11. Enhanced product ion spectra of (A) d_8-Glut-3-MH and (B) d_8-Glut-3-MHAl arising from pressing Sauvignon Blanc grape berries in the presence of d_8-(E)-2-hexenal.

This HPLC-MS/MS investigation gave us evidence for the missing piece of the precursor puzzle in the formation of Glut-3-MH and ultimately 3-MH via the pathway shown in Figure 12. We have tentatively assigned d_8-Glut-3-MHAl for the first time, showing it to be an obvious intermediate in the formation of d_8-Glut-3MH as could be expected. The involvement of enzymes such as glutathione *S*-transferase (GST) in the conjugation step in juice is an open question but the fact that we also obtained the alcohol d_8-Glut-3-MH clearly highlights the role of grape berry reductases in the juice (*48–50*). The process for formation of Glut-3-MHAl would naturally occur in the intact grape berry too, most likely facilitated by GST enzymes (*51*), followed by enzymatic reduction to the more stable alcohol Glut-3-MH. Whether Glut-3-MHAl can be found in juices in any appreciable amounts remains to be determined, but this compound appears to represent another source of 3-MH which has not previously received attention.

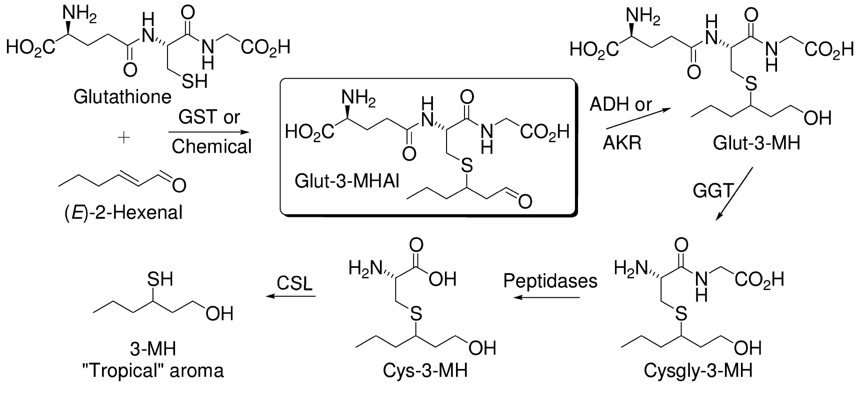

Figure 12. Formation pathway to 3-MH from GSH and (E)-2-hexenal, with a role for Glut-3-MHAl indicated for the first time. GST = glutathione S-transferase, ADH = alcohol dehydrogenase, AKR = aldo-keto reductase, GGT = γ-Glutamyltranspeptidase and CSL = carbon-sulfur lyase.

Summary

We have undertaken a great deal of work aimed at addressing aspects relating to 3-MH and its precursors in grape juices and wines. This included the development of a new SIDA method for the analysis of 3-MH which avoided the use of mercury and was designed for implementation on GC-MS instruments with a conventional EI source. We applied this method to a grape ripening study and identified free thiol 3-MH in grape juices for the first time. The method was also used for a survey of commercial Australian wines, showing that 3-MH may be an important aroma compound in varieties other than Sauvignon Blanc.

An array of precursor studies has provided some new insights into the relationship between Cys- and Glut-3-MH. Analysis of Sauvignon Blanc grapes during ripening revealed the presence of Cysgly-3-MH at trace levels and reinforced the importance of harvest timing for maximum precursor concentrations. Freezing studies highlighted the difference between frozen storage of juice and grape samples, where large amounts of Glut-3-MH were only formed when grapes were frozen, yet Cys-3-MH levels were not similarly affected. This indicated that loss of berry integrity could lead to Glut-3-MH formation and provided a warning that juices, but not grapes, could be frozen for later precursor analysis. Fining a juice with gelatin had minimal effect on precursor concentrations, while the effects of machine-harvesting and transportation of fruit were quite remarkable. While high levels of SO_2 minimized precursor formation, fruit transported after being commercially harvested had up to 10 times as much Cys-3-MH and around twice as much Glut-3-MH compared to the non-transported samples, revealing the ability to form precursors during processing. This was especially relevant since Cys-3-MH is more easily utilized by yeast during fermentation, and the levels we encountered from this experiment were the highest we have recorded for healthy fruit. Furthermore, Cysgly-3-MH was determined at around 100 nmol/L in the transported fruit samples, which was considerably more than any other samples we have assessed. However, as a transient intermediate, Cysgly-3-MH appears to be insignificant when compared to the concentrations of the other two precursor forms.

An experiment designed to inhibit enzymes usually active during berry crushing showed that concentrations of Glut-3-MH could be substantially reduced while Cys-3-MH levels were minimally affected. This suggests that Cys-3-MH, arising via Cysgly-3-MH from the breakdown of Glut-3-MH, is present in the grape berry and relatively resistant to short-term grape processing effects while the major portion of Glut-3-MH forms post-harvest so processing conditions can have a major influence on concentrations. As a way of rounding out these studies, we sought to understand the role of Glut-3-MHAl, the neglected intermediate in the formation of Glut-3-MH from GSH and (*E*)-2-hexenal. By adding labeled (*E*)-2-hexenal to Sauvignon Blanc grapes and then crushing them to obtain the juice we could identify labeled Glut-3-MHAl arising from conjugation of the added aldehyde with GSH naturally present in the berries. Not only that, the corresponding labeled Glut-3-MH was also present as a result of enzymatic reduction of labeled Glut-3-MHAl. This allowed us to fill in the gap between berry constituents GSH and (*E*)-2-hexenal and Glut-3-MH.

Our work has provided some unique insights into the formation and fate of precursors to 3-MH and emphasized their dynamic nature, which proceeded well beyond berry development and into the winemaking process. From the perspective of a winemaker, understanding the interrelationships between varietal aroma compound 3-MH and its conjugates has foreshadowed the potential to manipulate aspects of the winemaking process to optimize the varietal thiol profile of wines through control of precursor formation. Winemakers should be able to make quality improvements as a result of greater awareness about the effects of processing on aroma precursors.

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Chapter 3

Streamlined Analysis of Potent Odorants in Distilled Alcoholic Beverages: The Case of Tequila

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Sample dilution analysis (SDA) is a novel methodology for identifying key odorants in distilled alcoholic beverages by direct injection-gas chromatography-olfactometry (GCO). SDA has the potential to provide accurate key odorant identification with reduced analysis and material costs, while also reducing extraction bias. The methodology was applied to 100% agave *añejo* tequila, a spirit with distinct organoleptic qualities. Results from SDA and aroma extract dilution analysis (AEDA) were comparable, and identified 2/3-methyl-1-butanol, 2-phenylethanol, linalool, β-damascenone, guaiacol, 4-ethyl-guaiacol, eugenol, *trans*-isoeugenol, and vanillin as the key odorants of 100% agave *añejo* tequila.

Tequila is a distilled alcoholic beverage made from the fermented pulp of a particular species of agave, *Agave tequilana* Weber, in a geographically delimited area of Mexico. It is internationally recognized as a unique and important spirit and is considered to be culturally important and organoleptically distinct.

It has become common knowledge that tequila can exhibit a broad range of flavor characteristics – a search of the *New York Times* archives, for example, turns

In Flavor Chemistry of Wine and Other Alcoholic Beverages; Qian, M., et al.;
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up multiple tasting notes for high-end tequilas (*1–3*) – in much the same way that Scotch whisky does (*4*). Surprisingly, the modern flavor chemistry literature on the aroma components of tequila is quite sparse, with only a handful of research articles published in the last twenty years (*5–9*). Tequila's success as a distinctive product is due in large part to its unique organoleptic qualities. Therefore, it is an appropriate product for further investigation from the perspective of aroma composition and flavor chemistry.

Distilled alcoholic beverages like tequila offer a unique opportunity to streamline the aroma compound identification process. Unlike most food products, they are candidates for direct analysis by gas chromatographic (GC) methods. Most food products are not suitable for injection into a capillary column system: they are composed of lipids, proteins, and other non-volatiles that would irreversibly damage the equipment. Therefore, the aroma compounds of these foods must be extracted or isolated in a way that makes them available for GC analysis.

Whereas most foods must be extracted into solvents or sampled using headspace methods, tequila and other spirits are essentially already aroma extracts in an ethanolic matrix. Spirits as a class are usually 40-50% ethanol and 50-60% water, with the compounds of interest, which distinguish one type of alcoholic distillate from another – volatile aroma compounds and often non-volatile sugars, tannins, and other wood-extractives – making up less than 1% of the distillate by volume. With the proper GC parameters and, especially, inlet setup, spirits can be analyzed directly, without extraction or sampling.

This sort of non-extractive analysis has significant benefits. It helps to reduce the time required per analysis, which is important for developing high throughput analyses that can be used for industry or academic assays of alcoholic distillates. Direct analysis also helps to avoid a fundamental problem with sample preparation: extraction (or sampling) bias. Each extraction is, ideally, representative of the food as a whole, but the solvent (or adsorbent in the case of headspace sampling) will extract (or adsorb) compounds with different polarities, moieties, or volatilities differently, depending on its own properties. Thus, a solvent may over-extract certain compounds, leading to an overestimation of their importance to the food, or an adsorbent may have no affinity for a certain compound, so, no matter how important it is to the food, it will not be detected in subsequent analyses.

The problem of extraction bias is usually dealt with by making multiple extracts with different solvents or adsorbents, in order to account for a wide range of possible compounds, but this is time consuming and potentially expensive; avoiding an extraction step altogether is inarguably preferable. With alcoholic distillates, it is possible to inject the food product directly into a GC, the effluent from which can either be analyzed using olfactometric techniques (GCO) or using a mass spectrometer (GC-MS). In the present study tequila was used as a subject to test the possibilities for optimizing alcoholic distillate aroma characterization through the use of a non-extractive, direct-injection technique.

Objectives

- Demonstrate that direct analysis of distilled alcoholic beverages – like tequila – by GCO techniques presents an accurate, streamlined method for the identification of key aroma compounds.
- Identify the key aroma compounds of a 100% agave *añejo* tequila, an economically and culturally important product defined by its unique organoleptic qualities.

Tequila

History and Production

History

While production of a fermented, alcoholic beverage from agave – pulque – was known in Mexico prior to the arrival of European colonizers (*10*), "agave liquor" is thought to have been first produced in the country in the middle of the 16th century (*11*). Essentially modern tequila was being produced in the country by the early 17th century (*11*), and the industry has developed into one of the most powerful agricultural sectors in the country (*12*).

Tequila is a Geographical Indication (GI) – that is, the name "tequila" is linked to a geographical location within which the liquor must be produced (*12*): in this case the entirety of the state of Jalisco, as well as parts of the states of Guanajuato, Michoacán, Nayarit, and Tamaulipas. The idea of GI originates from Europe, specifically from the *Appellation d'Origine Contrôlée* of France, which links organoleptic qualities to physical and cultural geography (*13*). Tequila is the oldest GI outside of Europe, dating to 1974 (*12*). The United States agreed on a standard of identiy for tequila in the 1973 (*14*), and the European Union recognized tequila's GI in 1997 (*15*), indicating that tequila must have specific and unique organoleptic qualities.

Production

Tequila is an alcoholic distillate of the fermented product of the stem or *piña* of a single species of agave, *Agave tequilana* Weber or the "blue agave". To produce tequila, the *piña* is stripped of its leaves and baked or autoclaved to convert its polysaccharides (mainly inulin) into fermentable monosaccharides (fructose and glucose). A fermented wort can be produced from either pure agave mash, in which case the eventual tequila is "100% agave," or up to 49% other sugar sources can be added (usually cane), in which case the product is "*mixto*" tequila. 100% agave tequila is generally considered to be of distinct and superior organoleptic quality.

Fermentation usually takes place in large steel vats, after which the wort is twice-distilled to produce raw tequila. Before bottling, tequila can be aged in oak. Tequila which is bottled without wood-aging is *blanco* or silver tequila; tequila mixed with wood-aged tequila, caramel, oak-extracts, and glycerin at no more than 1% (v/v) is gold tequila (*16*) (this type of tequila is almost exclusively made from a *mixto* base); tequila which is aged in oak for at least 2 months is *reposado* or aged tequila; and tequila which is aged in oak for at least 1 year is *añejo* or extra-aged tequila (*5*). Tequila types are distinguished in Mexican law according to volumetric measures of total esters (*16*, *17*).

Compositional Analysis

Flavor

Tequila is a product of economic importance for which the organoleptic profile is critical. It is surprising, therefore, that there have been relatively few studies published on its flavor compounds and their sensory importance.

Benn and Peppard (*6*) identified a total of 175 components in a dichloromethane extract of gold tequila using GC coupled to a number of detectors, including flame ionization (FID), mass spectrometry (MS), and sulfur chemiluminescence. Gas chromatography-olfactometry (GCO) combined with aroma extract dilution analysis (AEDA) identified 60 important odorant peaks in the extract, of which 30 could be paired with identified peaks from the instrumental work. Using AEDA, the researchers identified *β*-damascenone, 2/3-methyl-1-butanol, 2/3-methyl-1-butanal, 2-phenylethanol, and vanillin as the key odorants of the tequila, but, unfortunately, attempts at creating an aroma model from the AEDA data were unsuccessful. Because gold tequila is almost always *mixto* and has significant flavor additives, it is likely that even a complete aroma model based on such a tequila would not have been representative of tequila as a category (*18*).

López (*7*) and López and Dufour (*8*, *9*) used GCO and Charm Analysis to identify the most potent odorants in 100% agave tequilas, including samples of *blanco*, *reposado*, and *añejo* tequila. The researchers found differences among the most potent compounds in aged and unagedd tequilas; among the most potent odorants, the researchers identified phenylethanol and phenylethyl acetate in the *blanco*, the same compounds with the addition of vanillin in the *reposado*, and phenylethanol, vanillin, and an unknown compound in the *añejo*.

While mostly concerned with authentification, Bauer-Christoph *et al* (*19*) demonstrated that ratios of 2/3-methyl-1-butanol differed between *mixto* and 100% agave tequilas; variations in these important odorants lend credence to the widely held conclusion that these are not interchangeable products. Peña-Alvarez *et al* (*20*) showed that, of 3 varieties of agave, *Agave tequilana* Weber had the highest number of terpenes; the same research group identified and quantified some of these terpenes in 100% agave tequila and mezcal itself (*5*). Vallejo-Cordoba *et al* (*16*) identified a number of volatile compounds which are known to be odor-active using solid-phase microextraction (SPME), with a focus on using ester concentrations to better elucidate tequila categorization.

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Vallejo-Cordoba and Gonzalez-Cordova (*21*) used an electronic nose array paired with SPME-GC-MS to successfully differentiate between tequila, and the related agave-derived distilled spirits mezcal, Sotol, and bacanora. Unfortunately from a flavor-chemistry perspective, many of these studies did not use any GCO or sensory techniques to confirm the sensory effects of the observed chemical differences to the flavor of tequila.

Many studies identify volatile compounds in tequila without confirming their importance to the actual flavor profile of tequila (*5*, *16*, *20*, *22–24*). Only Benn and Peppard's 1996 study (*6*) actually attempted to link compound identification and GCO work. Thus, it is readily apparent that tequila, especially 100% agave tequila, is a product for which further flavor work is necessary and appropriate.

Authenticity

Most of the volatile analysis work on tequila has focused on authenticity, rather than flavor analysis. In part this reflects the burgeoning market for tequila and its economic importance, and in part it reflects the difficulty involved in policing GI products like tequila (*12*) which are of such high economic value and produced over such a large range (*18*).

Using SPME and gas chromatography-isotope ratio mass spectrometry (GC-IRMS), Aguilar-Cisneros *et al* (*25*) found that they were generally not able to differentiate between types (*mixto* and 100% agave) and ages (*blanco*, gold, *reposado* and *añejo*) of tequila, and that even attempting to differentiate between tequila and other beverages was not always successful. On the other hand, Bauer-Christoph *et al* (*19*) used the same method with more success to discriminate between *mixto* and 100% agave tequilas. Vallejo-Cordoba *et al* found that concentrations of ethyl esters by SPME could be used to discriminate between the previously mentioned age-types of tequila (*16*). Surprisingly, Lachenmeier *et al* (*22*), while they confirmed that *mixto* and 100% agave tequilas could be discriminated by volatile concentrations, did not find the same result for other Mexican agave spirits, such as mezcal, sotol, or bacanora. López (*18*) found that stable isotope assays allowed determination of the plant origin in tequilas, thus establishing authenticity. In general, it appears that a number of chromatographic techniques, often paired with stable isotopic assays, allow satisfactory confirmation of tequila authenticity, but that work remains to be done to effectively differentiate tequila from other 100% agave distilled products.

Analysis of Potent Odorants in Spirits

Methods for Volatile Isolation

In general, the volatile compounds of a food are responsible for the characteristic aroma of that product (*26–29*), but it is important to note that not all volatile compounds contribute significantly – or, indeed, at all – to the aroma of a product. Before it is possible to determine which volatile compounds are significant, it is necessary to isolate these volatile compounds from the food matrix in which they exist. While there are multiple methods of volatile isolation, the most relevant to alcoholic beverage analysis are solid-phase microextraction (SPME) and solvent extraction (*28*, *29*).

SPME is solvent-free method of volatile isolation – a fiber, coated with adsorbent material, is exposed either to the headspace of a food product or, in some applications, is submerged directly into a liquid product (*30*). SPME offers reproducibility, high throughput with the possibility of automation, and reduces laboratory use of environmentally-damaging or health-hazardous solvents. It has been applied with some success to analysis of other distilled beverages (*30*, *31*), and has been used to analyze volatiles – not as odorants – in tequila (*5*, *25*). Unfortunately, SPME is also expensive, and the competitive behavior of compounds towards adsorption and with different coatings can result in failure to obtain a representative volatile sample of the food product (*29*).

As a method of obtaining a volatile isolate, solvent extraction relies on direct liquid-liquid extraction of a food product by an appropriate solvent. In alcoholic beverage analysis, the most common solvents used are diethyl ether, pentane, dichloromethane, and various Freons (chlorinated and fleuronated methanes) (*28*, *29*). One of the main considerations in alcoholic beverage extraction is limitation of ethanol extraction; ethanol is a volatile, odor-active compound, but is usually of limited interest to researchers. The use of highly non-polar solvents can help eliminate ethanol extraction. At the same time, attempts to limit ethanol extraction may limit the extraction of volatile compounds with similar properties; for this reason, it is often necessary to perform multiple extractions with different solvents in order to attempt to gain a fully representative isolate (*28*, *29*).

Thus, the weakness of producing a volatile isolate, whether by SPME or solvent extraction, is the introduction of bias into the isolate. Both adsorbents and solvents selectively affect the composition of volatile isolates. It is possible to correct this bias post-isolation, during quantification, using techniques like stable isotope dilution analysis (SIDA), but this technique cannot account for compounds that may be completely excluded from extraction, or compounds so volatile that they are entirely lost during extraction. Thus, it is clear that reducing or eliminating the need for isolation techniques during aroma analysis is prefereable whenever possible.

Direct Flavor Analysis of Distilled Alcoholic Beverages

As discussed above, it is usually necessary to isolate the volatiles of a food product before instrumental analysis of its key odorants. This is because gas chromatography, the main instrumental method for the analysis of aroma compounds from a food, can only process volatile compounds. If introduced into a GC, the lipids, proteins, and carbohydrates that make up the majority of the food matrix for most foods would interfere with analysis or even damage the column and chromatograph. Distilled alcoholic beverages, however, can in fact be thought of as pre-existing aroma extracts in an ethanolic solution.

Since this ethanolic solution is composed of ethanol – a volatile solvent – and water, given proper procedures for the introduction of the sample (*32*–*34*), direct injection of a distilled spirit, like tequila, into a gas chromatograph for analysis is feasible. However, because there is no concentration step applied to the volatile fraction, as usually happens in an isolation, it is necessary to inject a comparatively large amount of spirit in order to have detectable quantities of less-abundant volatile compounds. Thus, some consideration of appropriate injection technique is necessary.

There are two methods of sample introduction commonly used in aroma research: split/splitless and cool, on-column injection (*35*). Split/splitless inection allows for the injection of a relatively large sample, which is not necessarily clean; that is, the sample may contain some nonvolatile material that ideally should not be introduced to the column. During injection excess solvent can be vented, while the nonvolatiles in the sample remain in the inlet liner and are not transferred to the column. Unfortunately, the high temperatures required for this injection can break down labile compounds and form artifacts. Furthermore, heavier (less volatile) compounds are not transferred to the column as effectively as lighter (more volatile) compounds, resulting in the introduction of an inlet bias to the analysis.

Cool, on-column injection, which involves introducing a small volume of sample directly into the column or pre-column, eliminates any bias by transferring all of the sample directly to the column. Unfortunately, because the sample is transferred completely, only small-volume injections are possible, and the sample must be exceedingly clean to avoid damage to the column from non-volatile contaminants. Thus, neither method is ideal for direct analysis of spirits like tequila, which require large-volume injections of potentially "dirty" sample.

Programmable Temperature Vaporizer (PTV) inlets offer a compromise between the sensitivity and accuracy of on-column injection and the robustness of split/splitless injection. PTV inlets can be set up to run in cold-splitless mode, which combines the preservation of labile compounds characteristic of cool, on-column injection with the large-volume injection possible in split/splitless mode. This allows for large volume injection with relatively good (85-90%) transfer and no artifact formation (*36*). Direct injection of alcoholic beverages – with or without PTV inlets – for chromatographic analysis has been examined in several recent studies.

Da Porto *et al* (*34*) used direct injection *without* a PTV inlet, using hot split injection, to evaluate a novel orange spirit using GC-MS. Unfortunately, due to their lack of appropriate injection protocol, excessive ethanol transferred to the column and affected retention indices, making compound identification difficult. There is also potential concern with artifact formation due to lack of temperature control during the injection event. Madrera *et al* (*33*) used direct injection with more success, again without inlet temperature programming, to analyze both major and minor constituents of a cider distillate by GC-MS. Macnamara *et al* (*32*), using direct injection and a PTV inlet, showed that accurate identification and quantification of volatiles using cold-splitless direct injection of whiskey was possible.

As with the majority of the extant body of tequila volatile research, however, these studies all culminated in GC-MS identification of *all* volatiles. Without GCO techniques for both identification of odor activity and more sophisticated techniques like dilution analyses for selection of key odorants, it is impossible to say whether direct injection can present a streamlined analysis technique for the identification of potent odorants in distilled beverages.

Streamlined GCO Analysis

Dilution analysis is a technique that was developed for use with GCO in order to identify potent odorants and odor activity values (OAVs) without going through the laborious work of determining thresholds of all compounds involved. Two main types of dilution analysis are in use today: Charm Analysis, a technique pioneered by Dr. Terry Acree at Cornell University, and aroma extract dilution analysis (AEDA), a technique developed by Dr. Werner Grosch (*37*, *38*). The use of dilution analysis with GCO for distilled alcoholic beverages has been previously reviewed by Plutowska and Wardencki (*29*).

Both techniques proceed by serial dilution: a sample – generally a volatile isolate – is diluted serially and analyzed by GCO, with the GCO operator noting for how many dilutions each individual aroma peak persists. In AEDA, the number of dilutions in which a particular compound is detected is called its flavor dilution (FD) value or factor. The FD value of a compound is directly proportional to its OAV. In general, the compounds identified with the highest FD values in AEDA are considered most likely to be important contributors to the aroma of a food (*38*). The procedures can both be adapted for SPME, usually by decreasing the headspace or the amount of sample exposed to the headspace (*29*).

AEDA has been used successfully to reconstruct aroma models of alcoholic beverages (*39*). It has also been used, unsuccessfully, to attempt to reconstruct the aroma profile of a *mixto* tequila (*6*). With modern methods and some care, it should be possible to use direct injection, as described above, to streamline the dilution analysis of tequila. This research will demonstrate a streamlined process for the identification of key odorants in distilled alcoholic beverages.

Performance Evaluation of Direct Injection Technique

In order to ensure that streamlined analysis using PTV inlets does not result in increased inlet bias, a standard mix of seven different compounds was prepared to compare differences in transfer between direct, cold-splitless and cool, on-column injections methods.

Materials and Methods

The compounds used were 3-methyl-1-butanol, guaiacol, 2-phenylethanol, *cis*-whiskey lactone, *trans*-whiskey lactone, syringol, and vanillin. These compounds represent a range of moieties, polarities, molecular weights, and volatilities; they are also frequently cited as important aroma compounds in distilled alcoholic beverages, including tequila (*4*, *6*, *29*). Compounds were prepared in 10 ppm and 100 ppm solutions in diethyl ether, in order to determine if dilution affected transfer. The compound 3-methyl-1-butanol, which is known to be relatively unaffected by inlet bias due to its stability, volatility, and low molecular weight, was used as an internal standard.

Three injection protocols were compared using a CIS4 PTV inlet (Gerstel, Germany) using the following injection methods: hot-splitless, cold-splitless, and cool, on-column. Hot-splitless was added as a mode to show that some modes of injection are demonstrably worse for transfer or injection bias (*35*). For hot-splitless (1 min purge valve-delay), the inlet was maintained at 250 °C. For cold-splitless (1 min purge valve-delay), the inlet temperature was programmed from -50 °C (0.1 min hold time), then ramped to 250 °C at 12 °C/s. For cool, on-column injection the inlet temperature was programmed as follows: 40 °C initial temperature with +3 °C oven temperature tracking. Analyses were preformed using a 6890 GC (Agilent Technologies, Inc., Palo Alto, CA) equipped with an FID (250 °C) and RTX-WAX column (15 m x 0.53 mm i.d. x 0.5 µm film; Restek, Bellefonte, PA). Helium was the carrier gas at a constant flow rate of 5 mL/min. GC oven temperature was programmed from 40 to 225 °C at a rate of 10 °C/min with initial and final hold times of 5 and 20 min respectively.

Duplicate runs for both 10 ppm and 100 ppm concentrations were performed in order to obtain statistical validation. Relative abundance of each compound was calculated by normalizing peak areas against the peak area of 3-methyl-1-butanol. Statistics were calculatd using statistical functions of Microsoft Excel 2011 (Redmond, WA).

Results and Discussion

The comparison of injection methods showed no significant differences in compound transfer (performance) between cold-splitless and cool, on-column injection methods (see figure 1). In fact, while the differences did not exceed two standard deviations, the use of cold-splitless injection accomplished better compound transfer than either of the other methods for all compounds at the 100 ppm concentration level. At 10 ppm, minor differences between cold-splitless and cool, on-colum injection emerged for the transfer of vanillin and syringol, but this was not surprising, as they are among the least volatile compounds found in distilled alcoholic beverages. These differences, however, were not considered likely to impact any GCO dilution analyses, because the differences between the two injection methods were so much slighter than the usual dilution steps (e.g. 1:2, 1:3) found in the literature (*28*).

Potent Odorants in an Añejo Tequila

A 100% agave *añejo* tequila was subjected to both streamlined dilution analysis (SDA) using direct, cold-splitless injection and standard AEDA. This served two purposes. First, running side by side analyses allows a comparative evaluation of SDA as a method to identify potent odorants in alcoholic beverages as compared to AEDA, which is widely used enough to be considered a "gold standard" (*29*, *39*). Second, this work contributes to the surprisingly sparse literature on the aroma composition of tequila (see above), and is among only a handful of papers using modern flavor chemistry techniques to analyse a 100% agave *añejo* tequila (*6–9*, *21*, *40*).

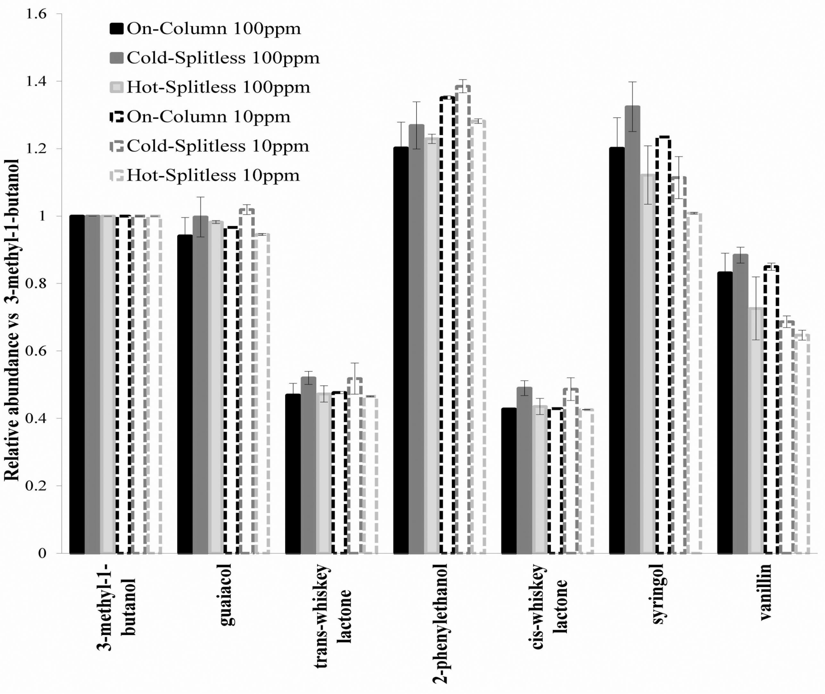

Figure 1. Injection performance comparison for selected volatile constituents of distilled alcoholic beverages. All peak areas were normalized against the corresponding peak area of 3-methyl-1-butanol. (Error bars represent standard deviations, n = 2.)

Materials and Methods

The tequila chosen for analysis was a 100% blue agave *añejo* tequila (Leyenda del Milagro *Añejo*, Tequilera Milagro S.A. DE C.V.: Mexico), purchased at a local liquor retailer. It had an ethanol concentration of 40% (v/v).

For sample dilution analysis (SDA), the tequila was diluted stepwise in 1:3 increments with absolute ethanol. AEDA was performed on stepwise (1:3 v/v) dilutions (in dichloromethane) of an extract prepared by liquid-liquid continuous extraction (LLCE). Flavor dilution (FD) factors were based on the highest dilution at which an odorant was detected GCO.

The LLCE extract was prepared as follows: 150 mL of tequila plus 400 mL of deodorized water was placed in an LLCE apparatus (part no. Z562440; Sigma-Aldrich, St. Louis, MO) equipped with condenser (4 °C) and a 250-mL flask containing 150 mL of dichloromethane. The dichloromethane was refluxed and the extraction conducted for 18 h. The solvent extract was dried over anhydrous sodium sulfate (20 g) and concentrated by distillation (Vigreux) to 5.5 mL. Note: the final concentration represented a ~27X enrichment of the original tequila (from 150 mL to 5.5 mL).

GCO was conducted using an Agilent 6890 GC equipped with an FID (250 °C) and a Gerstel OD2 sniff port. Injections were made in the cold splitless mode as described earlier. Separations were performed on both RTX-WAX and RTX5 columns (15 m x 0.53 mm i.d. x 0.5 µm film; Restek, Bellefonte, PA). Helium was the carrier gas at a constant flow rate of 5 mL/min. GC oven temperature was programmed from 40 to 225 °C at a rate of 10 °C/min with initial and final hold times of 5 and 20 min respectively. GC-MS analysis was performed for compound identification purposes as previously described (*41*). Criteria for compound identifications are described in the footnote of Table I.

Results and Discussion

Identification of Potent Odorants in 100% Agave Añejo Tequila

A total of 33 odor peaks were found in the tequila sample by GCO (Table I). Of these, 26 compounds were positively identified by comparison to the retention indices (RIs), odor impressions, and mass spectra of authentic standards (*41*). A further 2 compounds (*trans*-2-nonenal and sotolon) were tentatively identified according to the RI and odor impression of authentic standards. Both *trans*-2-nonenal and sotolon can be detected by the human nose below part-per-billion concentrations (*42*, *43*), making mass spectra difficult or impossible to obtain.

The most potent odorants in 100% agave *añejo* tequila, according to both SDA and AEDA were 2/3-methyl-1- butanol, 2-phenylethanol, linalool, *β*-damascenone, guaiacol, 4-ethyl-guaiacol, eugenol, *trans*-isoeugenol, and vanillin (Table I). These odorants all persisted until the last or second to last dilution in AEDA (FDs of 81 or 243). It is interesting to note that most of these compounds are also potent odorants in whiskeys (*4*, *39*). Since *añejo* tequila is aged in oak, as are whiskeys, it is likely that, as in whiskeys, the phenolic compouds are extracted from the wood, rather than originating in the agave *piñas*. The fusel alcohols are likely to be products of yeast metabolism that are present to some degree in all all distilled spirits. Linalool, however, is a terpene that is not thought to be important in wood-aged spirit aroma, and is likely to originate from the agave itself (*5*, *20*).

That is to say that all of these compounds have origins which can be traced to various steps of tequila production, whether it is the agave itself, the fermentation and distillation process, or the wood-aging of the *añejo* tequila. Furthermore, most of these compounds are known to be key impact odorants in other distilled beverages; therefore, it is reasonable to assume that they would play the same role in tequila. Linalool is the most interesting and unique compound to tequila (having also been identified by Benn and Peppard (*6*) and López (*8*, *9*) as a reasonably significant odorant). It is known to have citrus, herbal, and floral impressions, all of which are commonly used as descriptors for tequila (*1*–*3*).

Table I. Comparsion of Sample Dilution Analysis (SDA) and Aroma Extract Dilution Anslysis (AEDA) for the Detection of Predominant Odorants in an Añejo Tequila

no.	*compound*	*Odor description*[a]	*RI*[b]		*FD-factor*[c]	
			WAX	*RTX5*	*SDA*	*AEDA*
1	acetaldehyde[d]	sweet, pungent	<800	<500	<3	n.d.[f]
2	ethyl propanoate[d]	fruity, apple	935	745	n.d.	27
3	ethyl 2-methylpropanoate[d]	fruity	976	774	n.d.	3
4	2,3-butanedione[d]	buttery, cream cheese	986	618	<3	27
5	ethyl butanoate[d]	fruity	1047	807	n.d.	3
6	ethyl 2-methylbutanoate[d]	fruity	1063	856	n.d.	<3
7	ethyl 3-methylbutanoate[d]	fruity	1076	856	n.d.	9
8	2-methyl-1-propanol[d]	dark chocolate, malty	1101	641	<3	27
9	*Unknown*	fruity	1197	- -[g]	n.d.	3
10	2/3-methyl-1-butanol[d]	dark chocolate, malty	1211	751	27	243
11	ethyl hexanoate[d]	fruity	1243	1000	n.d.	3
12	acetic acid[d]	vinegar, pungent	1447	648	9	27
13	*trans*-2-nonenal[e]	hay-like, fatty	1508	1166	n.d.	3
14	linalool[d]	floral	1542	1100	<3	81
15	butanoic acid[d]	cheesy, fecal	1619	- -	<3	27
16	phenylacetaldehyde[d]	floral, rosy, plastic	1637	- -	n.d.	<3
17	3-methylbutanoic acid[d]	sweaty	1663	- -	<3	27
18	*Unknown*	hay-like, saffron	1722	1335	n.d.	9
19	β-damascenone[d]	floral, applesauce	1812	1391	9	81
20	guaiacol[d]	smoky	1848	1090	3	81
21	*trans*-whiskey lactone[d]	coconut	1877	1290	<3	27
22	2-phenylethanol[d]	rosy, wine-like	1891	1117	27	243
23	*cis*-whiskey lactone[d]	coconut, floral	1949	1325	3	9

Continued on next page.

Table I. (Continued). Comparsion of Sample Dilution Analysis (SDA) and Aroma Extract Dilution Anslysis (AEDA) for the Detection of Predominant Odorants in an Añejo Tequila

no.	*compound*	*Odor description*[a]	*RI* [b]		*FD-factor*[c]	
			WAX	*RTX5*	*SDA*	*AEDA*
24	4-ethyl guaiacol[d]	smoky, cloves	2019	1282	3	81
25	*unknown*	grape, sweet	2119	- -	n.d.	9
26	eugenol[d]	cloves	2153	1367	9	81
27	3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolon)[e]	curry, spicy	2173	- -	3	27
28	2,6-dimethoxyphenol[d]	smoky	2243	- -	3	27
29	*unknown*	woody, incense	2257	- -	n.d.	3
30	*trans*-isoeugenol[d]	smoky, cloves	2326	1458	<3	81
31	vanillin[d]	vanilla	2524	1403	3	81
32	ethyl vanillate[d]	vanilla, smoky	2602	- -	n.d.	<3
33	syringaldehyde[d]	smoky, vanilla	2845	- -	<3	<3

[a] Odor characteristics perceived during GCO. [b] Retention index calculated from GCO data. [c] Flavor dilution (FD) factor determined using RTX-WAX column. [d] Compound positively identified by comparison of its RI values, odor characteristics and mass spectra with those of an authentic standard. [e] Compound tentatively identified by comparison of its RI values and odor characteristics with those of an authentic standard. [f] n.d. = not detected. [g] - - = not available

Two main directions remain for further research. On the one hand, it would be interesting to apply the same exploratory technique to *blanco* and *reposado* tequila, to help determine which potent odorants in tequila are the products of wood-ageing and which originate from the agave. On the other, quantitation and model studies would allow verification of the accuracy of the current aroma analysis, and it is possible that sophisticated sensory studies could help direct further instrumental analyses.

Comparison of SDA and AEDA

When compared to AEDA, there were more compounds not detected (coded *n.d.* in Table I) in SDA (12 compounds) than in AEDA (1 compound). Furthermore, there was much less granularity in SDA, with a maximum FD of 27, than in AEDA, which had maximum FDs of 243. It is therefore apparent that the two methods are not necessarily equivalent.

However, it is worth recalling the two reasons for the development of SDA. First, the methodology was developed in order to implement streamlined analysis of alcoholic beverages (and other products) which are directly analyzable by

GC. Therefore, fewer compounds identified and lower FDs are not necessarily a negative result; in fact, if the important information provided through AEDA can also be obtained by SDA, at a lower time- and laboratory-cost, then it should be considered a successful method. Some examination of the results will show this to be the case.

The two compounds with the highest FDs (FD_{SDA} = 27) in SDA – 2/3-methyl-1-butanol and 2-phenylethanol – are also the two compounds with the highest FDs in AEDA (FD_{AEDA} = 243). In fact, all of the potent odorants identified by AEDA (compounds with FD_{AEDA} of 4 or 5) were also identified as potent odorants by SDA. Furthermore, only one potentially potent odorant was identified by AEDA and *not* by SDA: ethyl propanoate (FD_{AEDA} = 27).

Second, SDA is meant to potentially reduce bias due to extraction or isolation, which can result in the loss of volatile, labile, or poorly selected compounds (see above). Acetaldehyde, which is known to be present in alcoholic spirits in quantities likely to make it an odor impact compound (*44*), is better detected in these analyses by SDA than by AEDA. Because acetaldehyde is both highly volatile and highly labile, it is likely to be lost in extraction or isolation, explaining its absence in AEDA.

It is well-known that not all odorants identified by GCO are potent or impact odorants (*27*, *38*); in fact, the purpose of dilution methods, like AEDA, is to selectively identify only those compounds likely to contribute to the aroma of the actual product. It is demonstrated herein, however, that SDA and AEDA produce qualitatively similar data. In effect, SDA appears to skip the exhaustive step of identifying all *potential* key odorants and, instead, identifies *only* the key odorants. These data imply that SDA is able to deliver the same information as AEDA, while at the same time minimizing bias and avoiding time-consuming and expensive extractive and analytical work.

Of course, further work is necessary to verify these results. One avenue of productive research would be to construct tequila aroma models based on AEDA and SDA data, in order to confirm whether both (or either) were accurate reconstructions of the original product. It is also important to verify that SDA consistently produces results that mirror the key odorants identified by AEDA. Nevertheless, this research demonstrates that a streamlined dilution analysis (SDA) of distilled alcoholic spirits is both possible and potentially equivalent to existing, more labor- and material-intensive methods.

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Chapter 4

Assessing Smoke Taint in Grapes and Wine

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When wildfires or controlled burns occur in proximity to wine regions, there is potential for vineyard exposure to smoke and consequently, for ‘smoky’, ‘ashy’ sensory attributes, i.e. ‘smoke taint’, to appear in resultant wines. This paper concerns the quantification of glycoconjugates of guaiacol for the assessment of smoke taint in grapes and wine. In particular, this paper describes investigations into: (i) the provenance of guaiacol glycoconjugates in fruit from control (i.e. unsmoked) and smoke-affected grapevines; (ii) the metabolism of guaiacol glycoconjugates during fermentation; and (iii) the possible carry-over of smoke taint between growing seasons.

The volatile phenols, guaiacol and 4-methylguaiacol, have been used as marker compounds for the determination of smoke taint in grapes and wine, following grapevine exposure to smoke (*1–4*). These compounds are not considered to be solely responsible for smoke taint (*5*); in fact, their occurrence in wine is more commonly attributed to oak maturation (*6*). However, both guaiacol and 4-methylguaiacol have been identified as components of smoke (*7–9*), are known to exhibit ‘smoky’, ‘phenolish’, ‘sharp’ and ‘sweet’ aromas (*10*) and can be readily quantified by gas chromatography-mass spectrometry (GC-MS) using stable isotope dilution analysis (SIDA) methods (*6*).

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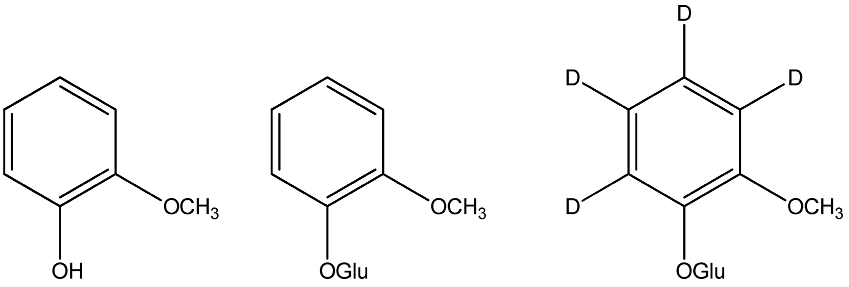
In experimental trials involving the application of smoke to grapevines, the concentrations of guaiacol and 4-methylguaiacol in wines were found to be indicative of the intensities of smoke-related sensory attributes (*3*, *4*). In contrast, the volatile phenol content of grapes has proven to be a less reliable indicator of the extent of grapevine exposure to smoke. The presence of guaiacol in grapes does not necessarily indicate the occurrence of smoke taint, since guaiacol has been identified as a natural component of several varieties of *Vitis vinifera*, including Merlot, Shiraz, Tempranillo and Grenache (*11*–*13*). Furthermore, since guaiacol has been shown to accumulate in smoke-affected grapes in glycoconjugate forms (*14*–*16*), the absence of free guaiacol does not ensure that grapes are unaffected by smoke taint. Kennison and coworkers reported trace (1 µg/L) levels of guaiacol in free-run juice from smoke-affected Merlot grapes and the subsequent evolution of smoke-derived volatile phenols, including guaiacol, during alcoholic and malolactic fermentation (*1*). This is likely attributable to the metabolism of guaiacol glycoconjugates by yeast and bacteria.

Dungey and colleagues hypothesized that glycosylation might constitute a physiological response of grapevines to smoke, to reduce the reactivity of smoke-derived volatile phenols (*16*). High performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis has enabled the tentative identification of several glycoconjugates of guaiacol to date, being: a glucoside, a glucose-glucose disaccharide, four glucose-pentose disaccharides and a rutinoside, of which the glucose-pentose disaccharides are the most abundant (*15*, *16*). The recent development of quantitative HPLC-MS/MS based SIDA methods, which use d_4-guaiacol β-D-glucopyranoside as an internal standard, for the determination of guaiacol glycoconjugates in grapes and wine (*16*, *17*), offers a more reliable approach to the assessment of smoke taint. Guaiacol **1** and its unlabeled and labeled β-D-glucopyranosides (**2** and **3**) are shown in Figure 1.



1
guaiacol

2
guaiacol β-D-glucopyranoside

3
d_4-guaiacol β-D-glucopyranoside

Figure 1. Guaiacol and its unlabeled and labeled β-D-glucopyranosides.

This paper describes the application of these methods to improve current knowledge regarding the chemistry of smoke taint. In particular, to investigate: (i) the accumulation of guaiacol glycoconjugates in grapes; (ii) the metabolism of guaiacol glycoconjugates during winemaking; and (iii) the potential for carry-over of smoke taint from one growing season to the next.

Accumulation of Guaiacol Glycoconjugates in Grapes

The guaiacol glycoconjugate content of control and smoke-affected grapes of several *Vitis vinifera* varieties were compared in order to investigate the natural abundance of glycosidic forms of guaiacol. Control (i.e. unsmoked) grapes were sourced from: Viognier and Merlot grapevines grown in vineyards located at the University of Adelaide's Waite campus in Glen Osmond, South Australia; Grenache grapevines grown in the Barossa Valley district of South Australia; and Chardonnay, Shiraz, Cabernet Sauvignon and Pinot Noir grapevines grown in the Adelaide Hills district of South Australia. Smoke-affected grapes were sourced either from field trials involving grapevine exposure to experimental smoke or from commercial vineyards in the Yarra Valley and Goulburn Valley districts of Victoria, which were exposed to smoke from bushfires that occurred between February 7 and March 14, 2009. Field trials involved enclosing grapevines in purpose-built smoke tents to enable the application of straw-derived smoke for 20 min, using experimental conditions described previously (*1*, *4*, *16*). Smoke treatments were applied at a phenological stage corresponding to approximately 7 days post-veraison.

Control and smoke-affected grapes were harvested at commercial maturity, i.e. at juice total soluble solids (TSS) levels of 23 ± 1 °Brix; with the exception of smoke-affected Cabernet Sauvignon and control Pinot Noir and Chardonnay which were harvested 'early' (i.e. at TSS of 19 ± 1 °Brix) and smoke-affected Shiraz, which was harvested 'late' (i.e. at TSS > 30 °Brix). The guaiacol glycoconjugate concentrations of grapes (as berry homogenate) were quantified by HPLC-MS/MS using the SIDA method developed by Dungey and colleagues (*16*); the results are shown in Figure 2.

All grape samples were found to contain guaiacol glycoconjugates. Control grapes contained relatively low levels of glycoconjugates, as natural grape components; with the exception of control Shiraz grapes, which contained considerably higher glycoconjugate levels compared with other varieties. However, this is consistent with previous research which reports guaiacol as a constituent of Shiraz grapes (*12*). The glycoconjugate levels of smoke-affected grapes were significantly higher, than for control grapes; even for short durations of smoke exposure, i.e. 20 min. For grapes exposed to experimental smoke, i.e. smoked Viognier, Grenache and Merlot grapes, similar glycoconjugate concentrations were observed, being 253, 294 and 358 µg/kg, respectively. The highest levels, i.e. between 875 and 1526 µg/kg, were found in grapes exposed to bushfire smoke. This is almost certainly a function of the duration of smoke exposure, which has previously been shown to influence the intensity of smoke taint in wine (*3*).

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Importantly, control and smoke-affected grapes could be clearly differentiated according to their guaiacol glycoconjugate content. As such, analytical methods which determine glycoconjugate concentrations offer promise as diagnostic tools for assessing smoke taint in grapes; albeit, analysis of a broader range of grape varieties and a larger sample number is required to fully characterize the natural abundance of guaiacol in free and glycoconjugate forms.

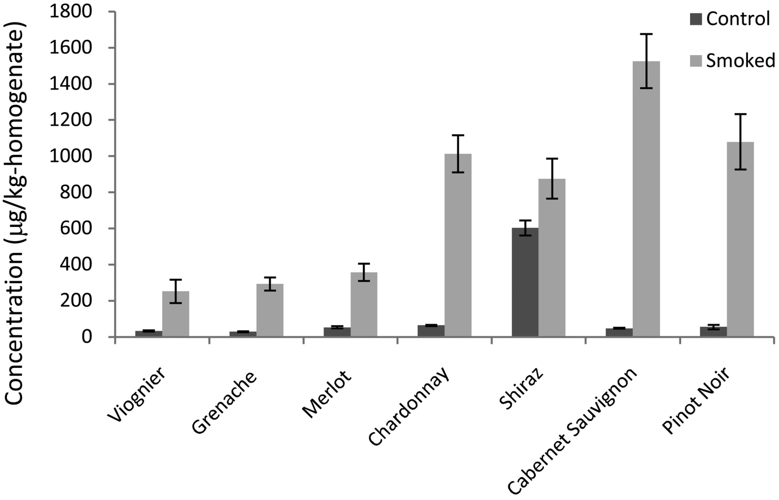

Figure 2. Concentration of guaiacol glycoconjugates in control and smoke-affected grapes of different varieties of Vitis vinifera. Viognier, Grenache and Merlot grapevines were exposed to experimental smoke; Chardonnay, Shiraz, Cabernet Sauvignon and Pinot Noir grapevines were exposed to bushfire smoke. Values represent means from three replicates ± standard error.

Metabolism of Guaiacol Glycoconjugates during Fermentation

To investigate the metabolism of glycoconjugates during winemaking, samples were collected at different time points throughout the fermentation of smoke-affected grapes. Grenache and Shiraz grapes were harvested from vines exposed to experimental and bushfire smoke respectively (as above), and fermented (in triplicate, on 50 and 5 kg scales respectively) according to standard small lot winemaking procedures, described elsewhere (*4*). Guaiacol glycoconjugate concentrations were again quantified by HPLC-MS/MS and the results are presented in Table 1.

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Table 1. Concentration of guaiacol glycoconjugates during fermentation of smoke-affected Grenache and Shiraz grapes

Sample	*Guaiacol Glycoconjugate Concentration*[a] *(µg/L)*	
	Grenache	*Shiraz*
grapes[b]	*420*	*1250*
free run juice	123 a	---
after 1 day maceration	197 b	---
after 3 days maceration	---	1027 a
after 4 days maceration	272 c	1112 a
after 7 days maceration	---	1025 a
after pressing	265 c	832 b
finished wine	290 c	825 b

[a] Values represent means from three replicates and were in agreement to ca. 10%. Values followed by a different letter within columns are significantly different ($P < 0.05$). [b] Berry homogenate concentrations were converted from µg/kg to µg/L based on 70% juice extraction rates (*18*).

For Grenache, glycoconjugate concentrations increased from 123 to 272 µg/L during alcoholic fermentation. This was likely due to the extraction of glycoconjugate from skins, since previous studies have demonstrated grape skin contains a higher proportion of guaiacol glycoconjugates than pulp (*16*). No significant changes in glycoconjugate levels were observed after pressing or following malolactic fermentation (i.e. in the finished wine). For Shiraz, glycoconjugate concentrations remained relatively constant across the sampling points applied during alcoholic fermentation, but a significant reduction was observed following pressing. This was attributed to the partial retention of glycoconjugates within grape marc, as reported previously (*16*), rather than metabolism by yeast. As observed for Grenache, glycoconjugate levels were not affected by malolactic fermentation.

For wines of both varieties, significant concentrations of glycoconjugates remained after winemaking: 290 µg/L for Grenache and 825 µg/L for Shiraz, being 69% and 66% of total grape glycoconjugates respectively; assuming 70% juice extraction (*18*). As in previous studies (*4*, *19*), these results indicate only a small proportion of the guaiacol glycoconjugate pool is metabolized by yeast, and metabolism by lactic acid bacteria is negligible. However, hydrolysis of glycoconjugates post-bottling would result in the intensification of smoke-related sensory attributes, and therefore smoke taint, in wine with ageing. This could explain the increased concentrations of guaiacol reported by Kennison and coworkers in smoke tainted wines after storage (*1*).

Descriptive sensory analysis (*20*) of the Grenache and Shiraz wines was performed using 12 trained panelists, to determine the intensity of a range of sensory attributes, including: 'fruit', 'smoke', 'cold ash', 'medicinal' and 'solvent' aromas and 'fruit' and 'smoky' flavors. The mean intensity ratings for each wine are shown in Figure 3. The panel considered the intensity of each sensory attribute to be higher in the Shiraz wine, as compared with the Grenache wine, but in particular, the 'smoke', 'cold ash' and 'medicinal' aroma attributes; i.e. attributes which have been previously associated with smoke tainted wines (*3*–*5*). Sensory analysis therefore confirmed the Shiraz wine to be more heavily tainted than the Grenache wine. Whilst this was to be expected given the different durations of smoke exposure, importantly, this finding demonstrates agreement between grape composition and the sensory properties of resultant wines; i.e. grape glycoconjugate content appears to be positively correlated with the intensity of smoke taint in wine.

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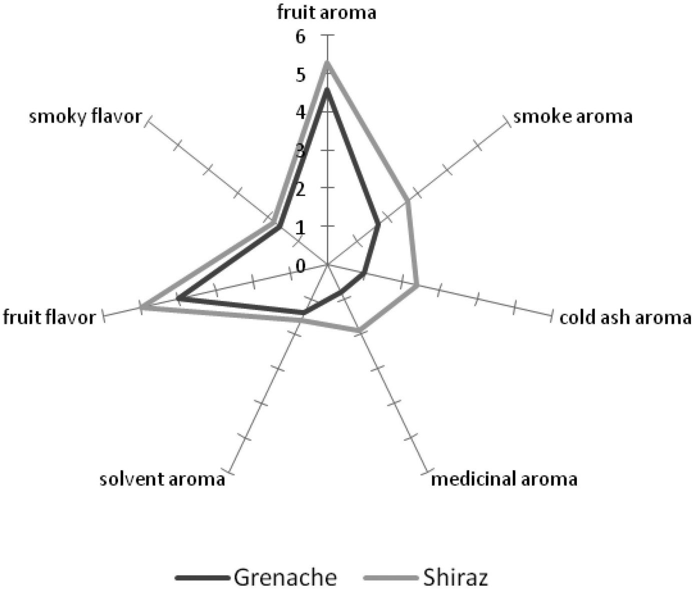

Figure 3. Mean ratings for sensory attributes of smoke-affected Grenache and Shiraz wines. Values represent mean scores from one wine replicate presented to 12 panelists in three replicate sessions. Data are from reference (4).

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Potential for Carry-Over of Smoke Taint between Seasons

Grapegrowers and winemakers have expressed concern that grapevine exposure to smoke may not only affect the composition and quality of fruit and wine produced during a particular growing season, but also the fruit and wine produced in the subsequent season; i.e. that there is potential for carry-over of smoke taint between seasons.

Grapevines store carbohydrate reserves in the woody tissues of the trunk and roots throughout the period of winter dormancy, i.e. until they are required to support the growth of new shoots and leaves during the early stages of the next growing season (*21*). This study aimed to establish whether or not smoke-derived guaiacol is similarly sequestered within the grapevine in glycoconjugate forms, and re-mobilized to bunches produced in the subsequent growing season.

The concentration of guaiacol glycoconjugates in grapes harvested from control (i.e. unsmoked) and smoke-affected Merlot and Viognier grapevines in the year in which smoke exposure occurred (i.e. Year 1) and in the subsequent growing season (i.e. Year 2, during which there was no smoke exposure) are shown in Figure 4.

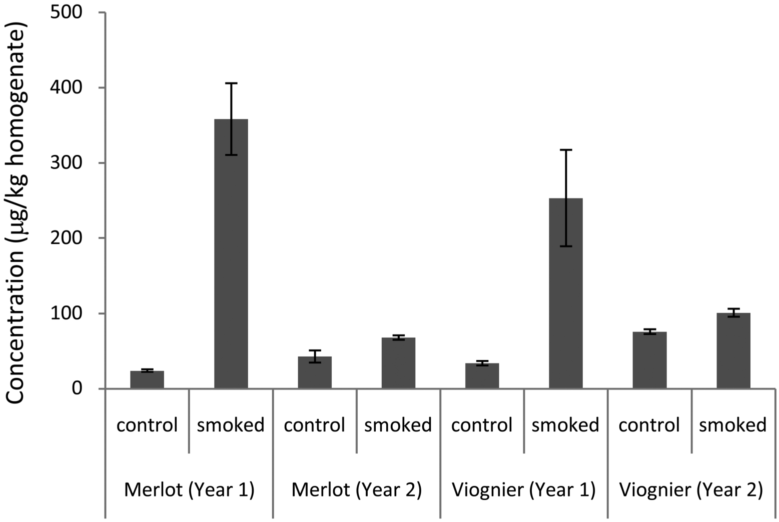

Figure 4. Concentration of guaiacol glycoconjugates in grapes harvested from control and smoke-affected Merlot and Viognier grapevines in the growing season during which smoke exposure occurred (Year 1) and in the subsequent growing season (Year 2). Values represent means from three replicates ± standard error.

As expected, in Year 1, grapes from smoke-affected vines contained significantly higher glycoconjugate levels than corresponding control grapes, irrespective of variety. However, there was no statistical difference between control grapes (Year 1 or Year 2) and grapes harvested from smoke-affected vines in Year 2. As such, there was no evidence to support the sequestration of guaiacol glycoconjugates prior to dormancy or any seasonal carry-over effect of smoke taint. These results are in agreement with previous findings. Kennison and coworkers reported significant reductions in the bunch number and crop yield of Merlot vines one year after repeated exposure to smoke; but only trace (2 µg/L) levels of guaiacol could be detected in the corresponding wines, indicating no long term impact on grape or wine composition (*22*).

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Conclusion

Quantification of guaiacol glycoconjugates offers a more reliable approach to the assessment of smoke taint in grapes and wine than quantification of free guaiacol. Determination of glycoconjugate concentrations enabled the differentiation of control and smoke-affected grapes. Additionally, the glycoconjugate content of wine can be used to assess the potential intensification of smoke taint with bottle age. It is hoped these findings will assist grape growers and winemakers to evaluate the extent of tainting following vineyard exposure to smoke.

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Chapter 5

Smoke Taint Aroma Assessment in 2008 California Grape Harvest

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In 2008, unprecedented wildfires in California, most notably in Northern California, caused considerable concern within the winemaking industry. Mendocino County, home to approximately 300 vineyards and 50 wineries experienced significant exposure. Pyrolysis of wood components during wildfires generates a mixture of volatile compounds. Volatile phenols such as guaiacol and 4-methylguaiacol contribute to smoke-derived aroma, and are reported as principal chemical markers in smoke tainted grapes. Our study confirmed that smoke taint marker compounds, guaiacol and 4-methylguaiacol, are bound to sugar in exposed berries. The glucose bound compounds will likely be released by yeast glucosidase during fermentation, whereas those that are non-glucose bound may be released by acid hydrolysis throughout wine aging in the bottle. An enzymatic hydrolysis method was developed to liberate the bound smoke-derived aroma and to quantify them via gas chromatography head space analysis. The proximity, intensity, and duration of wildfires were shown to correlate with the levels of glycosidic conjugates of guaiacol and 4-methylguaiacol in grapes.

In Flavor Chemistry of Wine and Other Alcoholic Beverages; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Introduction

Wildfires are a natural feature of the California environment and occur annually. In 2008, there was an unprecedented number of wildfires in California as shown in Figure 1. Somewhere over 3000 blazes occurred in just the first month of summer and provoked worry in the wine industry, as some of the wildfires were in close proximity to vineyards important for premium wine production. Mendocino County, home to about 300 vineyards that cover nearly 7,000 hectares, and 50 wineries experienced significant exposure to smoke. The negative impact of wildfires can also occur in areas not directly threatened by fire due to the effects of smoke (*1*).

In Australia, smoke from bushfires has been shown to directly impact grape composition and subsequent wine quality (*2*). In these studies, wines made from smoke-tainted fruit were described as having smoky, dirtly, earthy, burnt, smoked meat, damp fire, and ashtray aroma characteristics (*2*). Several molecules have been identified that have aroma characteristics similar to those used to describe wines made from smoke-tainted fruit (*2*). In previous work (*2*), guaiacol and 4-methylguaiacol have been used as “marker” compounds to quantify the extent to which fruit and wines have been affected by smoke (*1–3*).

Guaiacol and 4-methylguiacol are lignin degradation products (*5*, *6*). Pyrolysis of plant material during wildfires generates a complex mix of volatile organic compounds, including guaiacol and 4-methylguaiacol. These compounds can also be found in wines that have been aged in charred oak barrels (*7*). Guaiacol and 4-methylguaiacol also occur naturally in the fruit and leaves of many grape varieties (*8*, *9*).

Previous work (*8–10*) has proposed the concentration of these compounds increases during vinification due to the hydrolytic release of guaiacol and 4-methylguaiacol from their glycosylated forms. A further study (*11*, *12*) demonstrated that acid hydrolysis of wines provided evidence that bound volatiles act as reserves for guaiacol and 4-methylguaiacol, which are released during wine aging. These authors hypothesized the volatiles were most probably glycosidically bound. In this study, we used a novel enzymatic hydrolysis methodology to determine the concentrations of guaiacol and 4-methylguaiacol in fruit from various regions of Northern California with differeny proximity to wildfires. In addition using a novel enzymatic hydrolysis methodology we have identified the varying composition of sugars bound to guaiacol and 4-methylguaiacol.

Material and Methods

Grape Samples

Chardonnay and Cabernet Sauvignon fruits were sourced from selected vineyards located in Fresno, San Joaquin, Sonoma, and Mendocino counties in California following fire events in June 2008. For each vineyard, approximately twenty berry clusters were selected randomly throughout the block.

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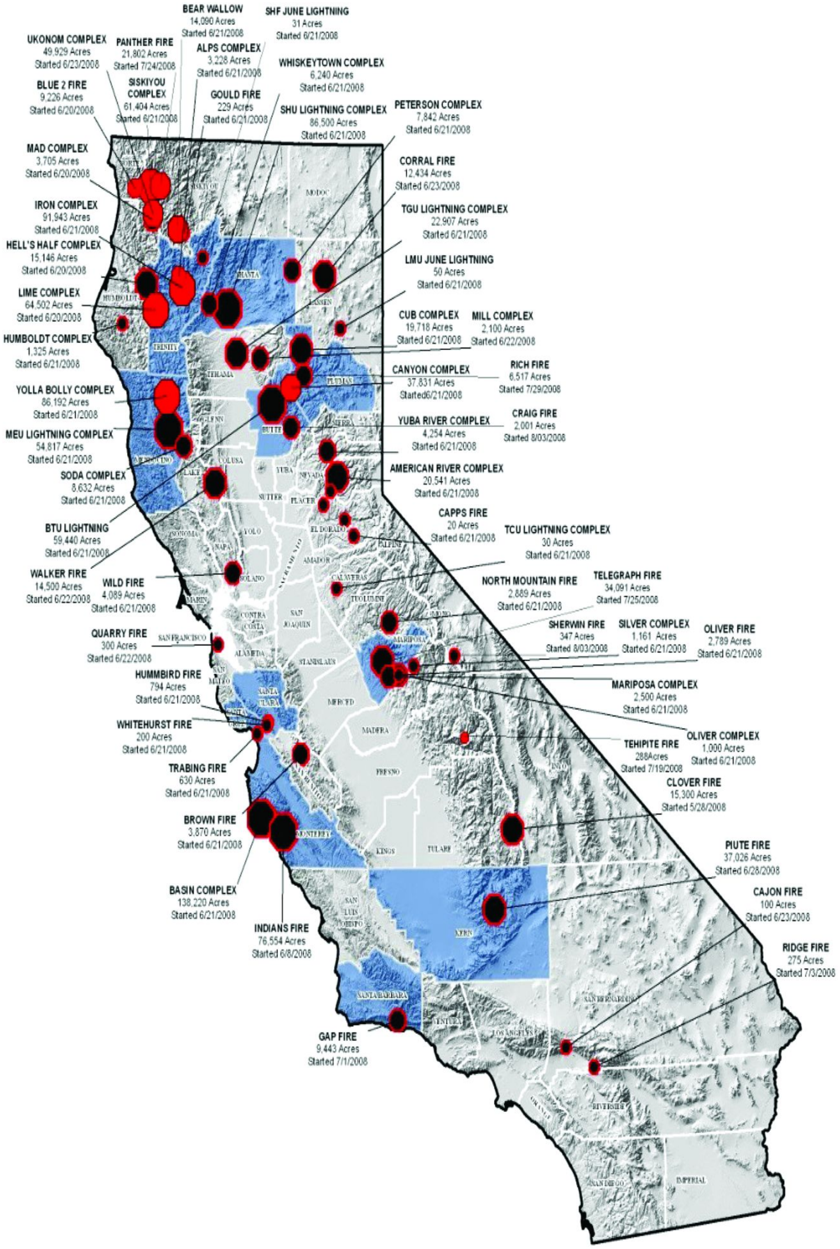
BEAR WALLOW
14,090 Acres
Started 6/21/2008
SHF JUNE LIGHTNING
31 Acres
Started 6/21/2008
UKONOM COMPLEX
49,929 Acres
Started 6/23/2008
PANTHER FIRE
21,802 Acres
Started 7/24/2008
ALPS COMPLEX
3,228 Acres
Started 6/21/2008
WHISKEYTOWN COMPLEX
6,240 Acres
Started 6/21/2008
BLUE 2 FIRE
9,226 Acres
Started 6/20/2008
SISKIYOU COMPLEX
61,404 Acres
Started 6/21/2008
GOULD FIRE
229 Acres
Started 6/21/2008
SHU LIGHTNING COMPLEX
86,500 Acres
Started 6/21/2008
PETERSON COMPLEX
7,842 Acres
Started 6/21/2008
MAD COMPLEX
3,705 Acres
Started 6/20/2008
CORRAL FIRE
12,434 Acres
Started 6/23/2008
IRON COMPLEX
91,943 Acres
Started 6/21/2008
TGU LIGHTNING COMPLEX
22,907 Acres
Started 6/21/2008
HELL'S HALF COMPLEX
15,146 Acres
Started 6/20/2008
LMU JUNE LIGHTNING
50 Acres
Started 6/21/2008
LIME COMPLEX
64,502 Acres
Started 6/20/2008
CUB COMPLEX
19,718 Acres
Started 6/21/2008
MILL COMPLEX
2,100 Acres
Started 6/22/2008
HUMBOLDT COMPLEX
1,325 Acres
Started 6/21/2008
CANYON COMPLEX
37,831 Acres
Started6/21/2008
RICH FIRE
6,517 Acres
Started 7/29/2008
YOLLA BOLLY COMPLEX
86,192 Acres
Started 6/21/2008
YUBA RIVER COMPLEX
4,254 Acres
Started 6/21/2008
CRAIG FIRE
2,001 Acres
Started 8/03/2008
MEU LIGHTNING COMPLEX
54,817 Acres
Started 6/21/2008
AMERICAN RIVER COMPLEX
20,541 Acres
Started 6/21/2008
SODA COMPLEX
8,632 Acres
Started 6/21/2008
CAPPS FIRE
20 Acres
Started 6/21/2008
BTU LIGHTNING
59,440 Acres
Started 6/21/2008
TCU LIGHTNING COMPLEX
30 Acres
Started 6/21/2008
WALKER FIRE
14,500 Acres
Started 6/22/2008
WILD FIRE
4,089 Acres
Started 6/21/2008
NORTH MOUNTAIN FIRE
2,889 Acres
Started 6/21/2008
TELEGRAPH FIRE
34,091 Acres
Started 7/25/2008
QUARRY FIRE
300 Acres
Started 6/22/2008
SHERWIN FIRE
347 Acres
Started 8/03/2008
SILVER COMPLEX
1,161 Acres
Started 6/21/2008
OLIVER FIRE
2,789 Acres
Started 6/21/2008
HUMMBIRD FIRE
794 Acres
Started 6/21/2008
MARIPOSA COMPLEX
2,500 Acres
Started 6/21/2008
WHITEHURST FIRE
200 Acres
Started 6/21/2008
OLIVER COMPLEX
1,000 Acres
Started 6/21/2008
TEHIPITE FIRE
288Acres
Started 7/19/2008
TRABING FIRE
630 Acres
Started 6/21/2008
CLOVER FIRE
15,300 Acres
Started 5/28/2008
BROWN FIRE
3,870 Acres
Started 6/21/2008
PIUTE FIRE
37,026 Acres
Started 6/28/2008
BASIN COMPLEX
138,220 Acres
Started 6/21/2008
CAJON FIRE
100 Acres
Started 6/23/2008
INDIANS FIRE
76,554 Acres
Started 6/8/2008
RIDGE FIRE
275 Acres
Started 7/3/2008
GAP FIRE
9,443 Acres
Started 7/1/2008
MODOC
SISKIYOU
SHASTA
LASSEN
HUMBOLDT
TRINITY
TEHAMA
PLUMAS
GLENN
BUTTE
SIERRA
MENDOCINO
COLUSA
YUBA
NEVADA
LAKE
SUTTER
PLACER
SONOMA
YOLO
EL DORADO
NAPA
ALPINE
AMADOR
SACRAMENTO
SOLANO
CALAVERAS
MARIN
TUOLUMNE
MONO
CONTRA COSTA
SAN JOAQUIN
SAN FRANCISCO
MARIPOSA
ALAMEDA
STANISLAUS
SAN MATEO
SANTA CLARA
MERCED
MADERA
FRESNO
INYO
MONTEREY
KINGS
TULARE
SAN LUIS OBISPO
KERN
SANTA BARBARA
VENTURA
LOS ANGELES
SAN BERNARDINO
RIVERSIDE
ORANGE
SAN DIEGO
IMPERIAL

Figure 1. The distribution of wildfires in California, 2008 (4). (see color insert)

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Sample Preparation

Berry clusters of Chardonnay and Cabernet Sauvignon were de-stemmed to get whole berries. These whole berries were then homogenized using a Retsch GM 200 Grinder (Retsch Inc., Haan, Germany). For free aroma analysis, 0.5 mL of 20% SDS solution was dispensed into 35 mL of homogenate followed by the addition of 14.5 mL of saturated sodium chloride solution to minimize enzymatic activity. After mixing, 10 g of treated homogenate was transferred into a GC headspace vial with septum cap (Microliter, Suwanee, GA). For bound aroma analysis, 50 mL homogenate was centrifuged for 10 min at 6,000 rpm. A total of 10 mL supernatant was mixed with 1.5 mL of 10M NaOH and then filtered through a 0.45 um syringe filter. The glycosylated bound aroma was isolated using Oasis HLB (hydrophilic, lipophilic balance) polymeric reverse phase extraction cartridge (*12*, *13*). At the end of the isolation process, a 1.2 mL eluent of bound aroma was transferred into a GC headspace vial.

Enzymatic Hydrolysis of Bound Aroma

For each vial that contains 1.2 mL eluent, 5.6 mL of 0.1 M citrate-phosphate buffer at pH 5 and 1 mL of enzyme cocktail were added. The enzyme cocktail comprised of 1 part each of Validase X, DP629, DP368, and DP423 (Valley Research Inc., South Bend, IN) plus Rohavin L (AB Enzymes, Darmstadt, Germany). These vials were capped then incubated at 45°C for 4 hours in a hot air oven.

Gas Chromatography Mass Spectrometry Analysis of Guaiacol and 4-Methylguaiacol

The free and bound forms of guaiacol and 4-methylguaiacol were analyzed by an Agilent 6890 series gas chromatograph equipped with 5973 series mass selective detector (Agilent Technologies, Palo Alto, CA). The column was a Stabilwax-DA capillary (0.25 mm I.D. x 30 m x 0.50 um film thickness; Restek, Bellefonte, PA). A Gerstel CIS 4 Inlet System and an autosampler (Gerstel, Baltimore, MD) were used for solid phase microextraction. A Supelco DVB/CAR/PDMS fiber (Sigma-Aldrich, St.Louis, MO) was exposed to the headspace of a sample vial for 20 min at 70°C to extract analytes before injecting into the GC front inlet at 60°C in a splitless mode with Helium as carrier gas. The oven temperature started at 50°C for 3 min, and then increased at a linear rate of 6°C/min to 150°C, continued with 20°C/min to final temperature at 240°C and held for 3 min. For data acquisition, the Selective Ion Mode was used and the ions monitored were m/z 81, 109, 124 for guaiacol and m/z 95,123,138 for 4-methylguaiacol.

Results and Discussion

Using the cited analytical methods below, the initial approach was to investigate whether smoke taint aroma compounds were present in free or bound form in Chardonnay berries exposed to smoke. Free guaiacol and 4-methylguaiacol were analyzed using SPME headspace GC/MS. Bound guaiacol and 4-methylguaiacol were analyzed using enzymatic hydrolysis followed by the same SPME headspace GC/MS method used for free guaiacol and 4-methyl guaiacol. Bound guaiacol and 4-methylguaiacol can be hydrolyzed by either acid or enzyme (*14–19*). Acid hydrolysis is believed to catalyze volatile compound transformation and can lead to potential under-estimation of smoke taint; therefore, enzyme hydrolysis is recommended for industrial application (*2*, *8*). Consequently, an enzyme hydrolysis method was adapted to quantify the bound smoke taint aroma compounds in the grape supply.

Structural studies have previoiusly elucidated that bound aroma compounds were glycosylated to sugars and were present mainly in the form of glucosides, arabinosyl glucosides, rutinosides, and apiosyl glucosides (*9*, *20*, *21*). It should be recognized that these glycosides were not fully hydrolyzed by enzymes during fermentation, and the increased concentration of free aroma compounds detected in finished wine during storage was due to acid hydrolysis of the remaining glycosides (*18*, *22–24*). This implication is especially important to the wine industry to ensure the quality of wine on the shelf. Consequently, it was important to consider the hydrolysis of all of these sugar moieties in order to obtain an accurate quantification of potential smoke taint.

The enzyme cocktail prepared in this study contained a wide spectrum of glycosidase activities (*β*-D-glucosidase, α-L-arabinofuranosidase, α-L-rhamnopyranosidase, and pectinases). The *β*-D-glucosidase activity was relatively more important due to a majority of known aroma compounds being bound to *β*-D-glucose (*20*). Results confirmed that smoke taint aroma compounds were almost entirely bound to sugar in exposed berries (Table 1). Many studies have explained that hydrophobic compounds are bound to sugar molecules to facilitate their transportation to the plant vacuole; thus, protecting the plant from any possible toxicity exhibited by an excess of hydrophobic compounds (*20*, *25*).

The enzyme hydrolysis method was applied to examine the extent of smoke impact to the grape supply. Results showed that Merlot grapes from Mendocino County vineyard had the highest concentration of guaiacol and 4-methylguaiacol (Figure 2). In comparison, Merlot grapes from vineyards in Fresno, San Joaquin, and Sonoma Counties, which were located further from major wildfires, had similarly low levels of both compounds. Therefore, the data would suggest that proximity of fire to the vineyard is a critical risk factor (Figure 3). This insight led to increased concern of the potential economic impact of wildfire because Mendocino County was a premium grape growing area (Table 2).

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Table 1. Analysis of smoke taint aroma compounds in berries from fire exposed Mendocino county Chardonnay vineyards

	Free Form[a] (ppb)		*Bound Form[a] (ppb)*	
Mendocino Vineyard	*Guaiacol*	*4-Methylguaiacol*	*Guaiacol*	*4-Methylguaiacol*
A	1.4	nd	4,134	3,485
B	1.4	nd	2,955	2,081
C	1.2	nd	1,973	1,506
D	nd	nd	2,009	1,138
E	nd	nd	2,311	2,060
F	nd	nd	2,474	1,828
G	nd	nd	2,450	1,524
H	nd	nd	2,327	1,612

[a] nd, not detected.

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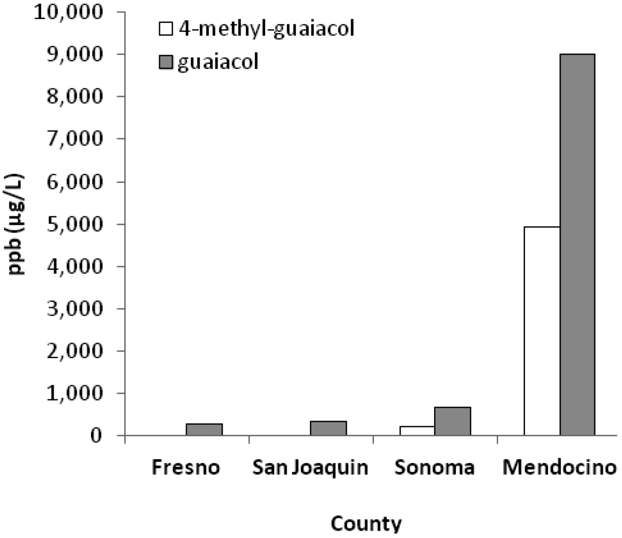

Figure 2. Concentration of guaiacol and 4-methylguaiacol in Merlot berries from 4 main grape growing counties in California. Berries were analyzed 3 months after the initial onset of wildfire in Mendocino County.

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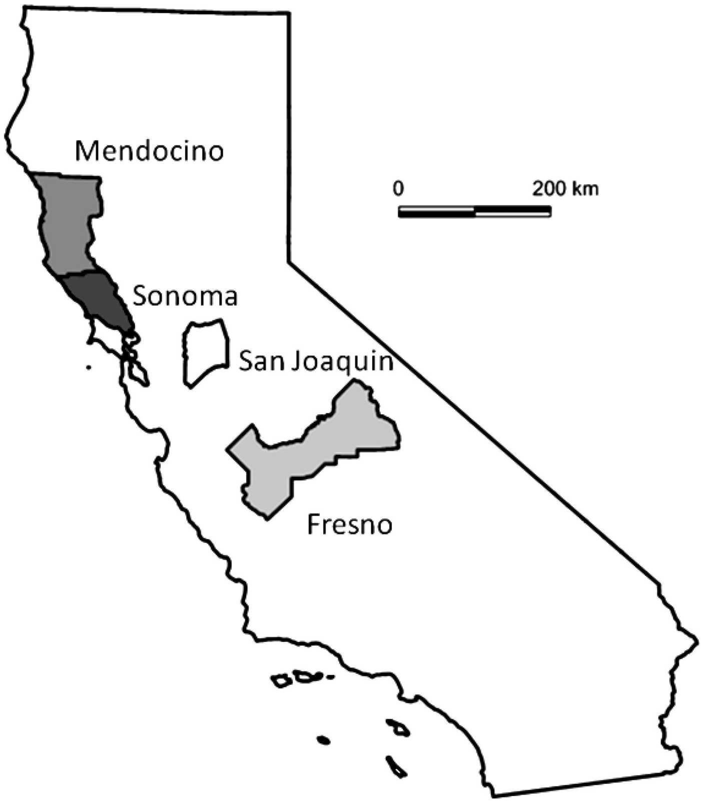

Figure 3. The location of the 4 counties in California.

Table 2. Merlot planted acreage and average price per ton by county in 2007*

	Fresno	*San Joaquin*	*Sonoma*	*Mendocino*
Hectares	529	3,295	2,781	749
Average price per ton	$236.13	$372.71	$1,452.25	$996.97

* California Department of Food and Agriculture (*26*).

Table 3. Types of Air Pollutants*

Pollutant
Carbon monoxide
Nitrogen dioxide
Ozone
Sulfur dioxide
Particulate matter with a median aerodynamic diameter less than 10 µm
Particulate matter with a median aerodynamic diameter less than 2.5 µm
Lead

* U.S Environmental Protection Agency (*27*).

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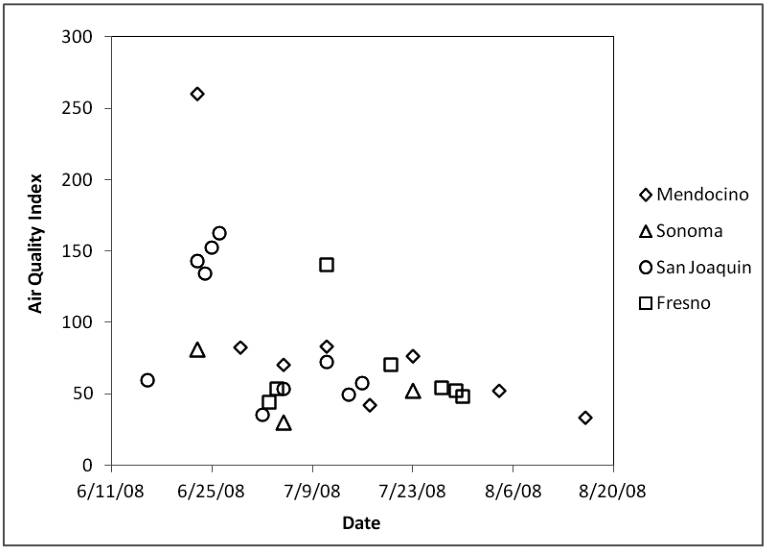

Figure 4. Air Quality Index (AQI) due to PM2.5 as the main pollutant for each County. An AQI ≤ 100 indicates a pollutant concentration that should not cause adverse health effects for most people; An AQI >100 indicates a pollutant concentration that may cause adverse health effects. (U.S Environmental Protection Agency) (27).

The United States Environmental Protection Agency has developed an "Air Quality Index" as part of its regulation of the Clean Air Act (*27*). The Air Quality Index reports the ambient concentrations of the major air pollutants and (Table 3) is calculated daily using the highest concentration of each pollutant recorded that day. It has been well established that particulate matter is the result of thermal degradation of wood components (*28*). While Fresno, San Joaquin, and Sonoma Counties also experienced wildfires, vineyards in Mendocino County were exposed to higher levels of particulate matter ("smoke taint" molecules) over a longer time period than the other three counties (Figure 4). More importantly, the Air Quality Index of particulate matter correlates with the chemical analysis data (Figure 2). This correlation suggests that the duration and intensity of smoke are also significant risk factors.

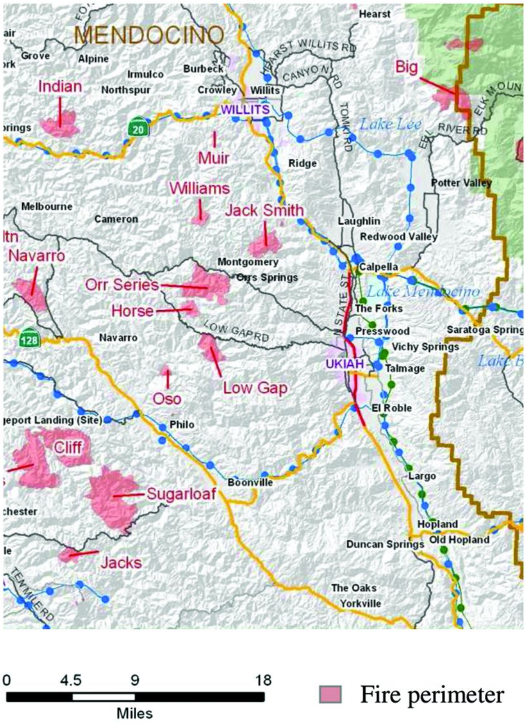
MENDOCINO
Hearst
Grove
Alpine
Irmulco
Burbeck
Northspur
Crowley
Willits
WILLITS
HEARST WILLITS RD
CANYON
TOMKI RD
Big
Lake Lee
EEL RIVER RD
ELK MOUN
Indian
20
Muir
Ridge
Williams
Jack Smith
Potter Valley
Melbourne
Cameron
Laughlin
Redwood Valley
Navarro
Montgomery
Orrs Springs
Orr Series
Calpella
Lake Mendocino
Horse
The Forks
128
Navarro
LOW GAP RD
N STATE ST
Presswood
Saratoga Spring
Vichy Springs
UKIAH
Talmage
Low Gap
Oso
El Roble
Bridgeport Landing (Site)
Philo
Cliff
Boonville
Largo
Sugarloaf
Hopland
Old Hopland
Duncan Springs
Jacks
The Oaks
Yorkville
TEN MILE RD
0
4.5
9
18
Miles
Fire perimeter

Figure 5. Mendocino Lightning Complex as of June 29th, 2008. (by California Office of Emergency Service) (29) (see color insert)

Early detection of smoke taint is crucial for the wine industry to forecast the grape supply risk level. As a result, Chardonnay and Merlot grapes from vineyards in the Ukiah area of Mendocino County were picked before harvest for analysis. These vineyards were within a 15 to 20 mile radius of major fires (Figure 5). Results showed that all samples, especially Merlot samples, contained significantly high levels of bound guaiacol and 4-methylguaiacol (Figure 6).

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Naturally occurring bound guaiacol was found in the juice of Merlot, Syrah, Tempranillo, and Grenache at a concentration range of 0.1 µg/L to 50 µg/L (*8*, *9*, *19*). Thus, the data would suggest that wildfire smoke significantly increased the concentrations of bound guaiacol and 4-methylguaiacol above the typical natural level in the grapes from vineyards in the Ukiah area of Mendocino County. Also, consistent with published findings in Australia (*2*, *10*), it was observed that guaiacol occurred at higher concentration than 4-methyguaiacol in smoke exposed samples. As for the difference in concentrations of bound guaiacol and 4-methylguaiacol between Merlot and Chardonnay, there is no direct explanation for this currently. The effect of smoke exposure on different varietals can be a subject for future research.

The detection thresholds for guaiacol and 4-methylguaiacol in red wines were 75 µg/L and 65 µg/L respectively; whereas, in white wines, the respective thresholds were 95 µg/L and 65 µg/L (*30*). A more recent study reported a much lower threshold for guaiacol, 30 µg/L, in a hydroalcoholic solution (*31*). Therefore, the abundance of precursors detected in Chardonnay and Merlot is far above threshold. This could lead to the production of unacceptable wines.

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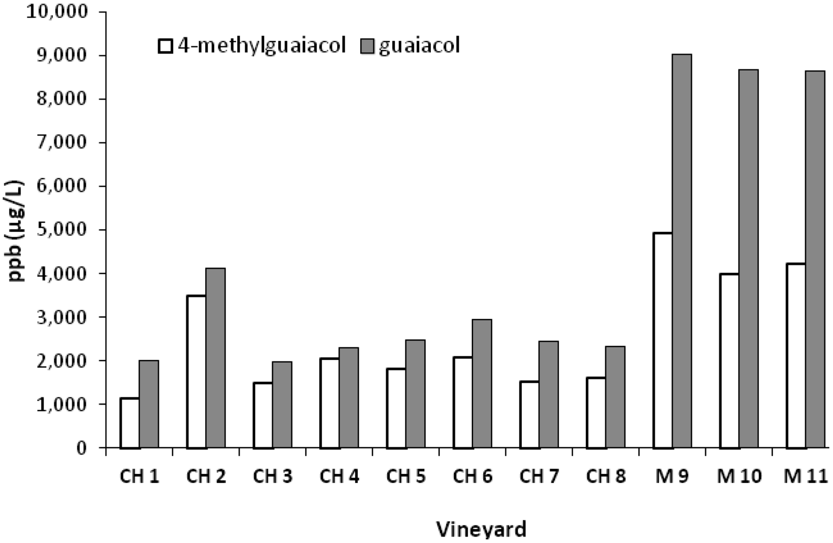

Figure 6. The level of bound smoke taint aroma compounds in berries from 11 different vineyards in Mendocino County. CH – Chardonnay; M – Merlot.

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Conclusions

As a result of wildfires, the 2008 grape supply from Mendocino County had elevated levels of glycoconjugated smoke taint aroma compounds compared to other grape growing regions of California. Mendocino County had a larger number and more intense wildfires than the other counties. The proximity of vineyards to the wildfires and the duration of the wildfires appear to have affected the amount of particulate material in the air. The quantity of particulate matter in the air correlated to the amount of guaiacol and 4-methylguaiacol found in grapes. Enzyme hydrolysis was used to quantify the concentration of bound guaiacol and 4-methylguaiacol in smoke exposed grapes. In the future, when wildfires occur near California grape growing regions, this enzymatic analysis can be used to assess smoke taint risk levels of the grape supply.

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Chapter 6

Development of C6 and Other Volatile Compounds in Pinot Noir Grapes Determined by Stir Bar Sorptive Extraction-GC-MS

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The development of volatile compounds in Pinot noir grapes during three growing seasons was investigated. Volatile compounds in grapes were analyzed using stir bar sorptive extraction-gas chromatography-mass spectrometry (SBSE-GC-MS) technique. The results showed that different compounds underwent different progressions during grape development. The C6 alcohols continuously decreased during the berry development except at very early stage. The C6 aldehyde, however, continued to accumulate until reaching to harvest maturity when they began to decrease. Most of the free monoterpene alcohols (geraniol, nerol and citronellol) only accumulated at the early grape development stage, and their concentration did not increase much at late stage of ripening, and some monoterpenes even decreased at later stage of ripening. Both β-ionone and vanillin increased only at very early stage of berry development, while benzyl alcohol and 2-phenylethyl alcohol dramatically increased over the whole growing season. There was little change observed for β-damascenone and γ-nonalactone during the ripening process.

Keywords: Aroma compounds; grape maturity; Pinot noir grape; stir bar sorptive extraction (SBSE)

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ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Introduction

Grape berry development process involves complex physical and chemical changes. These changes include berry volume expansion (and later shrinkage), structural changes of grape skin, pulp, and vascular tissue, switches in metabolic pathways, accumulation of sugars, breakdown of organic acids, and increase in pH (*1*).

Grape quality is of great importance to viticulturists and enologists because the final wine quality is largely determined by grape quality. Although sugar and acidity are frequently measured to assess grape quality, their contents in grapes are seldom related to wine quality. Volatile compounds in the grapes become the sought after alternative to assess grape quality. The evolution and accumulation volatile compounds in the grapes determine fruit quality (*2*).

Hundreds of volatiles have been reported in grapes, and some of them have been identified to be important contributors to grapes and wine quality. Volatile compounds in the grapes are the secondary metabolites of grapes berries, and they can be formed through many metabolic pathways such as mevalonic acid, shikimate, polyketide, and carotenoid breakdown pathways (*3*). Grape vvarieties differ greatly in their ability to produce the type and amount of volatile compounds, and these differences are responsible for the characteristic varietal aroma and flavor in the resulting wines. There are also substantial fruit-to-fruit variations within a variety due to differences in fruit location, growth temperature and sunlight, nutrition, harvest date (maturity), and post-harvest handling (*4*, *5*). However, it is still poorly understood how the agronomical conditions impact the volatile composition of grapes and the grape quality. Grape maturity is still one of the major concerns in the industry because it directly affects harvest decision.

Stir bar sportive extraction (SBSE) is a solventless sample preparation technique. Coated with poly(dimethylsiloxane) (PDMS), the stir bar can extract most volatile and semi-volatile compounds based on the partition coefficient between PDMS and aqueous solution. The SBSE technique has been proven to have lower detection and quantification limits compared with other conventional methodology (*6–8*). Coupled with GC-MS, the SBSE-GC-MS technique has been demonstrated to be very powerful to analyze trace amounts of volatile compounds in fruits (*9–13*) and wines (*14–16*).

Pinot noir originated in the Burgundy region of France, and has become popular in the United States, especially in Oregon. Recently, a preliminary sensory evaluation along with instrumental analysis showed that grape maturity (harvest date) significantly affected some key volatile compounds in wine (*16*). The wines from grapes harvested at late stage maturity contains higher concentration of C_{13}-norisoprenoids and monoterpenes than from the grapes harvested at early stage of maturity. However, it is still unknown if this difference comes from the grape volatiles or their glycoside precursors. In this experiment, we investigated the development of C6 aldehyde and alcohols and other volatile compounds during Pinot noir grape ripening. The information generated from this study added to the knowledge base of the formation of volatile compounds during berry development, and further help to understand the correlation between grape composition and wine quality.

Materials and Methods

Chemicals

All chemical standards and internal standards were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide was bought from J.T. Baker (Philipsburg, NJ), citric acid was from Staley Manufacturing Company (Decatur, IL), and sodium chloride was from VWR International (West Chester, PA).

Preparation of Standard and Internal Solutions

Citric acid buffer solution (0.2 M) was prepared by dissolving 42g of citric acid into 1L of Milli-Q water (Continental Water System, Millipore Corporation, Billerica, MA), and then adjusted to pH 3.1 using diluted sodium hydroxide solution. Standard stock solutions (about 1000 mg/L) were prepared in ethanol individually and stored at −15 °C. Before analysis, the standard stock solutions were diluted to the proper concentrations of working standards in the citric buffer. An internal standard solution was made by dissolving 1.93 ppm of octyl propanoate, 0.55 ppm of trans-carveol, and 0.94 ppm trans-2-nonenal in ethanol, and was stored at −15 °C.

Grape Sampling and Juice Preparation

Pinot noir grapes were grown at the Oregon State University experimental vineyard located in Alpine, OR. During the growing seasons of 2002, 2003 and 2004, ten clusters from different vines were randomly picked in the vineyard at different development stages and were immediately frozen at -29 °C. Berries were destemmed while still frozen, and then placed in a glass jar and kept at −23 °C. Prior to analysis, about 200 g of grape berries were thawed at 4 °C overnight and then ground using a commercial blender (Waring Products Division, New Hartford, CT). After settling for 5 min, skins and seeds were separated from the juice using cheese cloth, and then the grape juice was immediately analyzed. Separate grape juice was obtained by pressing the grapes and Brix and titratable acidity were measured.

Extraction of Volatiles in Grape Juice by Stir Bar Sorptive Extraction

The freshly prepared grape juice (10 ml) and 10 mL of 0.2 M citrate buffer (pH = 3.1) as well as 6 g of sodium chloride were mixed in a 40 mL vial, and 20 µL of internal standard solution was added. A pre-cleaned twister bar coated with PDMS phase (1 cm × 100 mm, Gerstel Inc., Baltimore, MD) was used to extract the volatile compounds from grape juices. The twister bar was constantly stirred for 3 hours at a speed of 1000 rpm. After extraction, the twister bar was rinsed with Milli-Q water, dried with tissue paper, and placed into autosampler tray (Gerstel Inc.).

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The extracted samples were analyzed using an Agilent 6890 GC-5973 MS system (Agilent Technologies, Little Falls, DE). The analytes were thermally desorbed in the thermal desorption unit (TDU) (Gerstel Inc.) in splitless mode, ramping from 35°C to 300 °C at a rate of 700 °C/min, and held at the final temperature for 3 min. The desorbed analytes were cryofocused (−80 °C) in a programmed temperature vaporizing (PTV) injector (CIS 4, Gerstel Inc.) with liquid nitrogen. Solvent vent injection mode was employed with a venting flow of 50 mL/min at 10 psi venting pressure for 0.01 min. After SBSE desorption, the PTV was heated from −60 °C to 250 °C at a rate of 10 °C/sec and kept at 250 °C. A ZB-FFAP capillary GC column (30m, 0.32mm ID, 0.25μm film thickness; Phenomenex, Torrance, CA) was employed to separate the analytes. The column carrier gas was helium at 2 mL/min. The oven temperature programmed initially at 40 °C (for 2 min), then increased at 6 °C/min to 180 °C, further increased at 4 °C/min to 240 °C, and held at the final temperature for 20 min. The electron impact (EI) energy was 70 eV, and the ion source temperature was set at 230 °C.

Calibration and Quantification of Volatile Compounds in Grape Juice

The stock solutions were prepared by dissolving ca 10,000 mg/L of each target compound individually into ethanol solution. Before analysis, certain amounts of stock solutions were added in synthetic juice to make the mixed standard solution and diluted with synthetic juice to give standard working solutions with a range of concentrations.

After adding 6g of sodium chloride and 20 μL of internal standard solution, the standard working solutions were extracted with PDMS stir bar for 3 hours. The SBSE extracts were than analyzed using the same procedure as described previously. The selected MS ions were used for quantification. Triplicate analysis was performed on all samples, and the average values are reported.

Results and Discussion

Since the efficiency of SBSE technique for volatile extraction is based on the equilibrium of analytes between PMDS solid phase and sample solution, the extraction of analytes was influenced by numerous factors (*17*). Several studies have been attempted to optimize SBSE extraction (*18–20*), however, the extraction efficiency is always sample and analyte dependent. During grape development, the pH of grape juice varied in a wide range, which could affect the extraction efficiency of the stir bar. Therefore, for quantification, 10 mL of buffer solution (pH=3.1) were mixed with grape juice to minimize the sample matrix effect. Moreover, 6 g of sodium chloride were also added to improve the extraction sensitivity.

Table 1. Standard curve and quantification of volatile compounds in grape juice

Compounds	*Quantify ion*	*Standard Correlation Coefficient*	*Compounds*	*Quantify ion*	*Standard Correlation Coefficient*
trans-Carveol (IS)	*109*		*2-Nonenal (IS)*	*70*	
Linalool	71	0.998	β-Damascenone	121	0.998
Nerol	69	0.997	β-Ionone	177	0.988
Geraniol	69	0.997	γ-Nonalactone	85	0.991
Eugenol	164	0.998	Vanillin	151	0.983
Citronellol	81	0.989	Hexanal	82	0.981
Linalool oxide[#]	94		*trans*-2-Hexenal	83	0.960
α-Terpineol	93	0.992	Heptanal	70	0.943
1-Hexanol	69	0.993	Octanal	84	0.996
Benzyl alcohol	108	0.996	Nonanal	98	0.974
Phenylethyl alcohol	122	0.999	Decanal	112	0.932
trans-3-Hexenol	82	0.994			
cis-3-Hexenol	82	0.994	*Octyl propinoate (IS)*	*112*	
trans-2-Hexenol	57	0.994	Methyl vanillate	151	0.987
1-Octen-3-ol	57	0.996	Ethyl vanillate	196	0.977
3-Methylbutanol	70	0.979			

[#] Linalool oxide was calculated based on the calibration curve of linalool.

A total of 27 volatile compounds including C6 alcohols and aldehydes, terpene alcohols, C_{13}-norisoprenoids and shikimic acid derivatives, were investigated in Pinot noir grapes during development. Target compounds were selected based on the results of the previous GC/Olfactometry studies of Oregon Pinot noir wines (*21*). In addition, monoterpene alcohols (linalool, linalool oxide, nerol, citronellol and α-terpineol), C_{13}-norisoprenoid (β-damascenone) and a few other volatile compounds (1-octen-3-ol, γ-nonalactone, vanillin, methyl and ethyl vanillate) were included in this study, since these compounds have been reported to be important to Pinot noir wine flavor. Acids, short carbon-chain alcohols, and ethyl acetate, were not quantified because SBSE cannot effectively extract polar compounds. Three different internal standards were used to quantify these compounds based on their similar physical and chemical properties. Table 1 listed the internal standards used for the quantification of corresponding volatile compounds. The correlation coefficients (R^2) of quantification for most compounds were greater than 0.99, and the relative standard deviations (RSD) were less than 15% for most of the quantified compounds (data not shown).

The Brix and titratable acidity for the grapes over eight weeks of development were illustrated in Figure 1.

As shown in the Figure 1, both TA and Brix varied widely among the three vintages. Year 2002 was a cool year at the grape growing season so the grape ripened late. However, the hot weather at the harvest time quickly pushed the grapes to commercial maturity. Year 2004 had cool weather during the harvest, and the brix was lower at harvest than 2002 and 2003.

The concentrations of volatile compounds in different stages of Pinot noir grape development during 2002, 2003 and 2004 were shown in Table 2, 3 and 4. The concentration of individual volatile compounds varied from vintage to vintage, reflecting the impact of climate on grape vine secondary metabolism.

Among the volatile compounds analyzed in the grapes, C6 alcohols (1-hexanol, *trans*-2-hexenol, *trans*-3-hexenol, and *cis*-3-hexenol) and aldehydes (1-hexanal and *trans*-2-hexenal) are well known as green and vegetable odorants (*22*). Among these green odorants, *trans*-2-hexenol was the most abundant compound in all the three years, showing a sharp increase after veraison and decreased in the late stage. 1-Hexanol, 1-hexanal and *trans*-2-hexenal all had the similar trend during grape development. Hexanal, *trans*-2-hexenal, as well as *trans*-2-hexenol in grapes can be converted to 1-hexanol during wine making (*23*), which also has a green aroma note. Therefore, wines from late harvest grapes could contain less 1-hexanol, which partially explains why they generally have less green and un-ripe aromas. However, yeast metabolism is very complicated; 1-hexanol can be converted to esters or oxidized

Cis- and *trans*-3-hexenol cannot be metabolized by wine yeasts, and generally will stay through fermentation (*23*). In all three vintages, a sharp decrease of *cis*-3-hexenol was observed after veraison. The grape samples had only a small amount of *trans*-3-hexenol (< 10 µg/L juice) and its decreasing trend was not obvious. Although *cis*-3-hexenol had lower sensory threshold than the *trans*-3-hexenol, aroma extract dilution analysis (AEDA) of Pinot noir wine showed that the *trans* form had higher flavor dilution values than the *cis* form (*21*), suggesting a higher concentration of *trans*-3-hexenol in the finished wine, possibly due to transformation of *cis*-3-hexenol to the *trans*-3-hexenol occurring during wine making process (*24*).

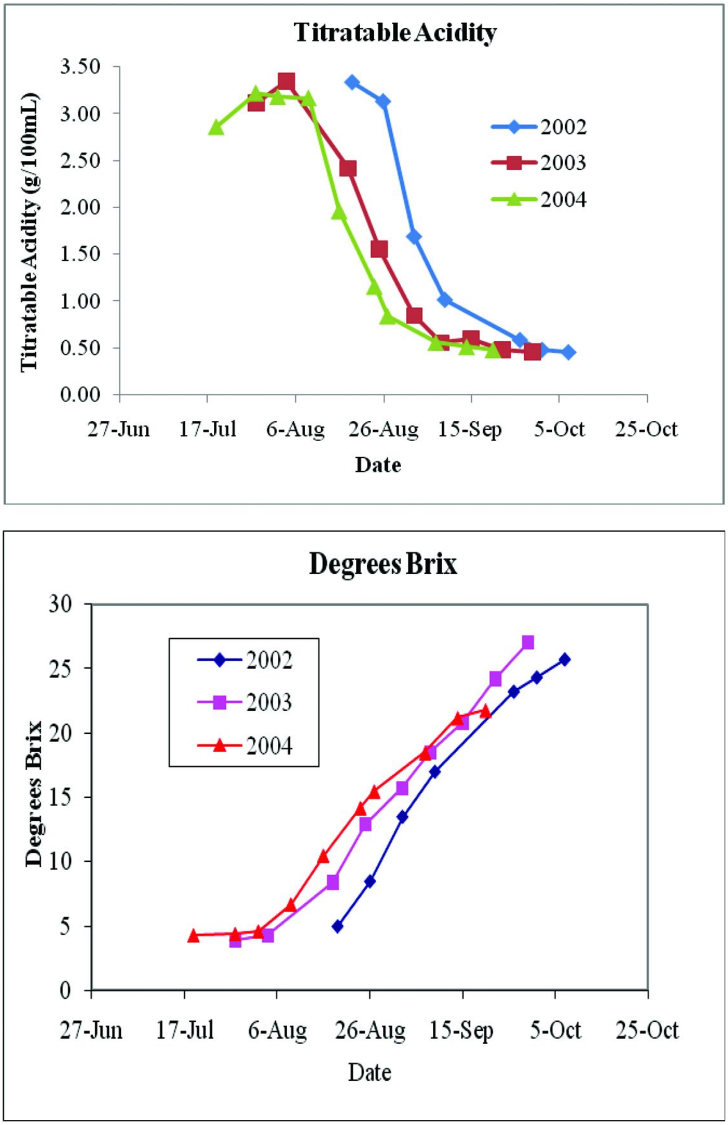

Figure 1. (top) The titratable acidity for the grapes over eight weeks of development in 2002, 2003 and 2004. (bottom) The Brix for the grapes over eight weeks of development in 2002, 2003 and 2004.

The total C6 alcohols and total C6 aldehydes during grape maturity were demonstrated in Figure 2 and Figure 3. Total C6 alcohols decreased during the grape development, whereas total C6 aldehyde only decrease at the final stage of grape ripening.

Monoterpenoid is another group of plant secondary metabolites, of which the biosynthesis begins with acetyl-coenzyme A (CoA). Monoterpene alcohols can exist in free form in the grapes and directly contribute to wine flavor. However, majority of terpene alcohols are present in grapes as glycosides, and these glycosides can be hydrolyzed to release the free terpene alcohols during wine making and aging process, and contribute to wine flavor. In addition, acid rearrangement of the monoterpene alcohols can occur under wine pH. For example, linalool can transform into α-terpineol, hydroxyl linalool, geraniol, and nerol under acidic condition (*25*). Among the terpenoids studied, geraniol had a higher concentration than others (Table 2, 3, 4), and it could be an important aroma contributor of wine due to its low sensory threshold (*21*). During all of the three vintages, the contents of geraniol, nerol, and citronellol increased in the early grape development stage, and remained constant at the late grape development stage. Similar trends were also observed with free terpene alcohols in Muscat de Frontignan (*26*). Only trace amounts of linalool, linalool oxide, and α-terpineol were detected in grape juices (<2 ppb), and they decreased along with grape development. The total amount of free terpene alcohols mirrored the individual terpene alcohol, the total amount of terpene alcohols increased in the early of grape development but decreased at late stage of grape development (Figure 4)

This result did not contradict our previous finding that the concentration of monoterpenes in wine increasing along with grape maturity (*16*). Free terpene alcohols are only present at a small portion in grape musts, and the majority of terpenes are present in grapes as glycosides, which could be hydrolyzed by enzymes and acid (*27*). Therefore, the bound form of monoterpenes should also be studied to fully understand their effects on wine.

β-Damascenone and β-ionone have been reported to be very important aroma-active compounds in wine (*28*, *29*). β-Damascenone has a general fruity aroma, and β-ionone has a berry note at low concentration and contribute to a raspberry note at higher concentration. Both compounds have low sensory threshold and contribute to wine aroma even below parts per billion. β-Damascenone had a concentration at low ppb level in the grape juice, β-ionone even had a lower concentration. During grape development, both β-damascenone and β-ionone had higher concentrations at the early grape development, but lower when the grape approached maturity. At the commercial maturity, their concentrations were the sample for the last three weeks, which is consistent with literature (*30*).

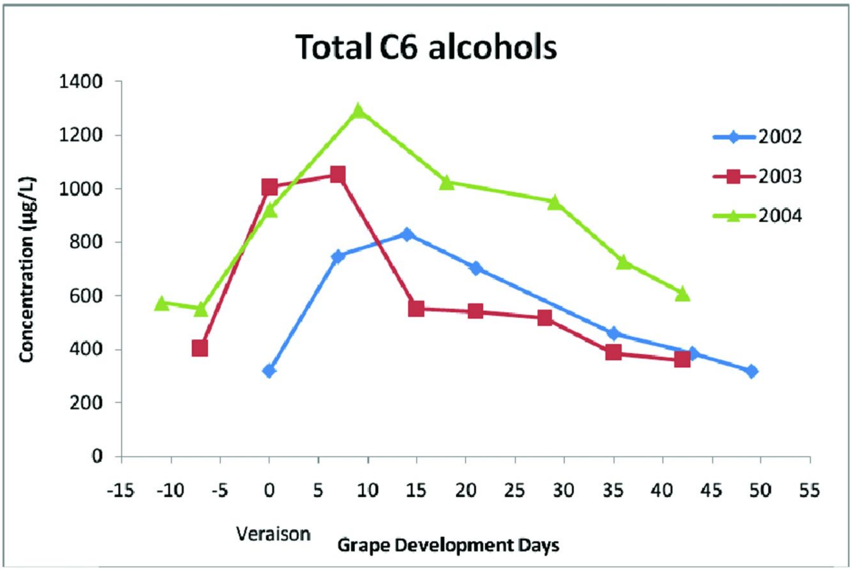

Figure 2. Total C6 alcohols during grape development.

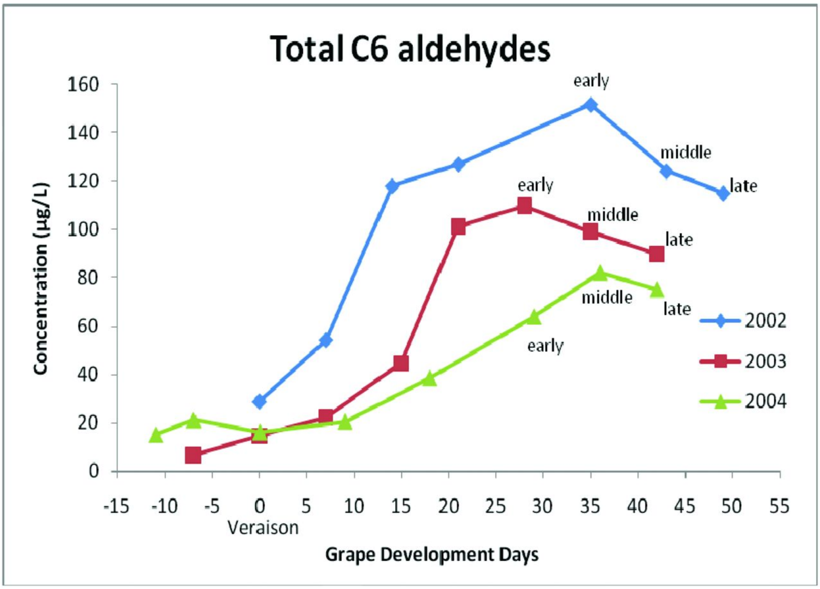

Figure 3. Total C6 aldehyde during grape development.

Table 2. Free volatile compounds in Pinot noir grapes during 2002 (μg/L juice)

	8/19/2002 (Veraison)	*8/26/2002*	*9/2/2002*	*9/9/2002*	*9/23/2002 (early harvest)*	*10/1/2002 (mid harvest)*	*10/7/2002 (late harvest)*
C6 alcohols and aldehydes							
1-Hexanol	24.9±0.1	32.9±0.1	59.9±0.1	83.2±0.1	78.0±0.1	61.7±0.1	51.1±0.1
trans-3-Hexenol	2.49±0.21	3.63±0.23	4.14±0.67	3.47±0.45	2.33±0.14	8.13±0.80	5.65±0.37
cis-3-Hexenol	190.1±1.0	413.4±0.7	421.8±0.6	241.6±0.7	52.4±0.5	25.5±0.6	25.8±0.5
trans-2-Hexenol	102±2	297±1	344±1	375±1	326±1	288±1	235±1
Hexanal	16.9±0.1	37.2±0.1	69.2±0.1	78.1±0.1	98.1±0.1	86.8±0.1	79.2±0.1
trans-2-Hexenal	4.5±0.1	10.1±0.1	42.5±0.1	45.3±0.1	50.3±0.1	34.5±0.1	33.5±0.1
Heptanal	1.56±0.01	1.65±0.01	1.85±0.01	1.38±0.01	1.37±0.01	1.09±0.02	1.08±0.01
Octanal	1.45±0.01	1.40±0.01	1.40±0.02	0.56±0.01	0.42±0.01	0.44±0.02	0.31±0.01
Nonanal	1.94±0.01	1.93±0.01	1.69±0.01	1.13±0.01	1.00±0.01	0.87±0.01	0.61±0.01
Decanal	2.48±0.01	1.97±0.01	1.31±0.01	0.55±0.01	0.50±0.01	0.39±0.01	0.25±0.01
Terpene alcohols							
Linalool	1.27±0.02	0.87±0.01	0.75±0.01	0.58±0.01	0.57±0.08	0.36±0.02	0.30±0.01
Nerol	1.17±0.01	1.02±0.02	1.84±0.02	2.51±0.02	3.62±0.02	3.74±0.01	2.50±0.01
Geraniol	2.26±0.01	3.01±0.01	7.66±0.01	8.94±0.01	10.78±0.01	8.35±0.01	7.54±0.01
Citronellol	0.72±0.01	0.95±0.01	1.76±0.01	1.59±0.01	1.77±0.01	1.06±0.01	1.17±0.01

Terpene alcohols							
Linalool oxide	0.86±0.03	0.42±0.02	0.11±0.02	0.06±0.03	0.06±0.02	0.09±0.05	0.06±0.03
α-Terpineol	0.45±0.01	0.31±0.01	0.22±0.01	0.17±0.01	0.13±0.01	0.12±0.01	0.12±0.01
C13-norisoprenoids							
β-Damascenone	0.33±0.01	0.47±0.02	0.91±0.01	0.27±0.01	0.12±0.02	0.07±0.01	0.07±0.01
β-Ionone (ng/L)	46.1±0.1	46.7±0.1	55.6±0.1	46.6±0.1	29.2±0.1	23.4±0.1	24.1±0.1
Others							
3-Methylbutanol	ND[*]	ND	88±10	130±5	300±12	375±11	573±5
Benzyl alcohol	141±1	91±6	254±3	525±3	1,367±6	1,742±8	1,824±7
Phenylethyl alcohol	52±5	52±2	119±2	212±3	305±4	335±5	565±7
γ-Nonalactone	0.20±0.01	0.25±0.01	0.18±0.01	0.35±0.01	0.20±0.01	0.22±0.01	0.23±0.01
Methyl vanillate	13.8±0.1	14.1±0.1	27.9±0.1	75.0±0.1	30.6±0.1	23.3±0.1	23.5±0.1
Ethyl vanillate	0.78±0.09	1.01±0.06	0.84±0.15	0.78±0.63	0.34±0.03	0.18±0.01	0.51±0.07

[*] ND: not detected

Table 3. Free volatile compounds in Pinot noir grapes during 2003 (µg/L juice)

	8/11/2003	*8/18/2003 (Veraison)*	*8/25/2003*	*9/2/2003*	*9/8/2003*	*9/15/2003 (early harvest)*	*9/22/2003 (mid harvest)*	*9/29/2003 (late harvest)*
C6 alcohols and aldehydes								
1-Hexanol	19.1±0.1	73.9±0.1	107.3±0.1	137.1±0.1	65.6±0.1	36.7±0.1	27.2±0.1	23.5±0.1
trans-3-Hexenol	3.16±0.17	2.38±0.55	3.94±0.28	4.14±0.35	5.39±0.57	5.78±0.15	6.30±0.36	6.92±0.80
cis-3-Hexenol	160.4±1.0	469.0±1.3	389.7±0.2	143.4±1.0	81.3±0.7	42.6±0.7	37.5±0.9	35.1±0.3
trans-2-Hexenol	221±2	463±2	554±1	552±1	541±1	432±1	316±1	294±1
Hexanal	3.6±0.1	9.4±0.1	13.3±0.1	26.2±0.1	58.2±0.1	65.6±0.1	53.5±0.1	53.7±0.1
trans-2-Hexenal	1.0±0.1	3.2±0.1	7.5±0.1	16.9±0.1	41.3±0.1	42.7±0.1	43.4±0.1	34.8±0.1
Heptanal	0.36±0.02	0.38±0.07	0.36±0.01	0.54±0.01	0.60±0.08	0.50±0.01	0.57±0.01	0.25±0.01
Octanal	0.34±0.02	0.31±0.01	0.28±0.03	0.27±0.01	0.26±0.01	0.22±0.01	0.58±0.01	0.35±0.01
Nonanal	0.73±0.01	0.69±0.01	0.47±0.01	0.49±0.01	0.58±0.01	0.46±0.01	0.58±0.01	0.53±0.01
Decanal	0.66±0.01	0.50±0.01	0.39±0.01	0.33±0.01	0.39±0.01	0.32±0.01	0.34±0.01	0.35±0.01
Terpene alcohols								
Linalool	0.80±0.03	0.77±0.01	0.71±0.03	0.53±0.02	0.54±0.02	0.53±0.02	0.54±0.01	0.49±0.02
Nerol	0.78±0.02	0.74±0.01	1.31±0.02	2.99±0.01	3.37±0.01	3.15±0.01	2.84±0.01	2.73±0.01
Geraniol	1.67±0.01	4.70±0.01	7.98±0.01	9.50±0.01	13.51±0.01	11.48±0.01	8.51±0.01	7.47±0.01
Citronellol	0.58±0.01	1.23±0.01	1.48±0.01	1.54±0.01	2.15±0.01	1.59±0.01	1.10±0.01	1.05±0.01

Terpene alcohols								
Linalool oxide	0.66±0.04	0.23±0.02	0.09±0.02	0.06±0.02	0.06±0.03	0.05±0.01	0.06±0.02	0.08±0.01
α-Terpineol	0.35±0.01	0.24±0.01	0.20±0.01	0.19±0.01	0.19±0.01	0.17±0.01	0.18±0.01	0.16±0.01
C13-norisoprenoids								
β-Damascenone	0.07±0.01	0.20±0.01	0.16±0.01	0.12±0.01	0.13±0.01	0.12±0.02	0.07±0.02	0.06±0.01
β-Ionone (ng/L)	28.7±0.1	44.0±0.1	37.7±0.1	36.7±0.1	32.5±0.1	30.1±0.1	23.2±0.1	24.2±0.1
Others								
3-Methylbutanol	41±19	45±17	72±9	182±14	202±5	254±4	425±6	512±4
Benzyl alcohol	61±4	79±5	249±6	766±6	1,074±3	1,382±3	1,436±8	1,379±3
Phenylethyl alcohol	24±3	35±2	127±2	264±6	318±3	325±4	361±5	496±1
γ-Nonalactone	0.29±0.01	0.21±0.01	0.11±0.01	0.17±0.01	0.13±0.01	0.13±0.01	0.27±0.01	0.33±0.01
Methyl vanillate	21.5±0.1	16.1±0.1	63.5±0.1	168.3±0.1	182.1±0.1	153.0±0.1	144.9±0.1	147.1±0.1
Ethyl vanillate	ND*	0.03±0.76	0.33±0.10	0.66±0.02	1.01±0.04	0.87±0.08	2.37±0.13	8.43±0.06

[*] ND: not detected

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Table 4. Free volatile compounds in Pinot noir grapes during 2004 (μg/L juice)

	7/28/2004	*8/2/2004*	*8/9/2004 (Veraison)*	*8/18/2004*	*8/27/2004*	*9/7/2004 (early harvest)*	*9/14/2004 (mid harvest)*	*9/20/2004 (late harvest)*
C6 alcohols and aldehydes								
1-Hexanol	34.5±0.1	30.3±0.1	49.3±0.1	105.0±0.1	86.3±0.1	91.7±0.1	90.2±0.1	85.4±0.1
trans-3-Hexenol	3.00±0.13	9.01±0.64	10.15±1.51	12.02±0.51	9.33±1.01	6.89±1.26	7.16±0.64	7.36±0.73
cis-3-Hexenol	201.5±1.4	170.0±1.1	284.6±0.5	485.1±0.6	177.2±1.2	75.0±1.7	39.5±0.9	35.4±0.3
trans-2-Hexenol	333±1	341±2	578±1	693±1	753±1	777±1	590±1	481±1
Hexanal	9.7±0.1	13.1±0.1	7.0±0.1	11.1±0.1	20.7±0.1	35.5±0.1	45.1±0.1	38.3±0.1
trans-2-Hexenal	3.3±0.1	5.8±0.1	6.4±0.1	8.1±0.1	16.2±0.1	26.6±0.1	35.7±0.1	35.6±0.1
Heptanal	0.21±0.01	0.23±0.01	0.51±0.06	0.44±0.02	0.39±0.03	0.41±0.07	0.36±0.01	0.27±0.01
Octanal	0.36±0.02	0.43±0.01	0.51±0.04	0.20±0.01	0.28±0.03	0.31±0.02	0.20±0.05	0.22±0.02
Nonanal	0.70±0.01	0.75±0.01	0.77±0.01	0.39±0.01	0.48±0.01	0.51±0.01	0.42±0.01	0.41±0.01
Decanal	0.81±0.01	0.82±0.01	0.98±0.01	0.36±0.01	0.44±0.01	0.54±0.01	0.30±0.01	0.28±0.01
Terpene alcohols								
Linalool	1.76±0.01	1.24±0.01	1.10±0.01	1.09±0.01	0.74±0.02	0.55±0.02	0.33±0.01	0.39±0.01
Nerol	1.36±0.01	1.26±0.01	1.05±0.02	1.63±0.02	3.71±0.02	3.91±0.01	3.67±0.02	3.57±0.02
Geraniol	4.30±0.01	2.36±0.01	3.35±0.01	7.94±0.01	15.79±0.01	15.03±0.01	13.07±0.02	13.07±0.01
Citronellol	0.46±0.01	0.38±0.02	0.64±0.01	1.56±0.01	2.20±0.01	2.24±0.01	1.72±0.01	1.94±0.01

Terpene alcohols								
Linalool oxide	1.02±0.01	0.77±0.01	0.45±0.02	0.31±0.02	0.10±0.03	0.08±0.04	0.10±0.02	0.08±0.05
α-Terpineol	0.63±0.01	0.46±0.01	0.44±0.01	0.38±0.01	0.22±0.01	0.19±0.01	0.11±0.01	0.11±0.01
C13-norisoprenoids								
β-Damascenone	0.05±0.01	0.05±0.01	0.13±0.01	0.11±0.01	0.12±0.01	0.07±0.01	0.04±0.01	0.05±0.02
β-Ionone (ng/L)	20.9±0.1	20.4±0.1	29.5±0.1	24.0±0.1	21.4±0.1	19.1±0.1	14.1±0.1	14.5±0.1
Others								
3-Methylbutanol	90±14	91±20	102±22	163±30	194±11	270±13	277±11	314±4
Benzyl alcohol	203±8	196±9	218±7	225±3	697±5	1,563±11	1,607±10	1,658±8
Phenylethyl alcohol	71±6	54±6	76±7	64±5	245±3	280±6	299±3	309±3
Methyl vanillate	27.8±0.1	17.7±0.1	23.1±0.1	33.9±0.1	75.2±0.1	128.4±0.1	104.5±0.1	102.5±0.1
Ethyl vanillate	ND[*]	ND	ND	ND	ND	0.19±0.02	0.16±0.01	0.27±0.04

[*] ND: not detected

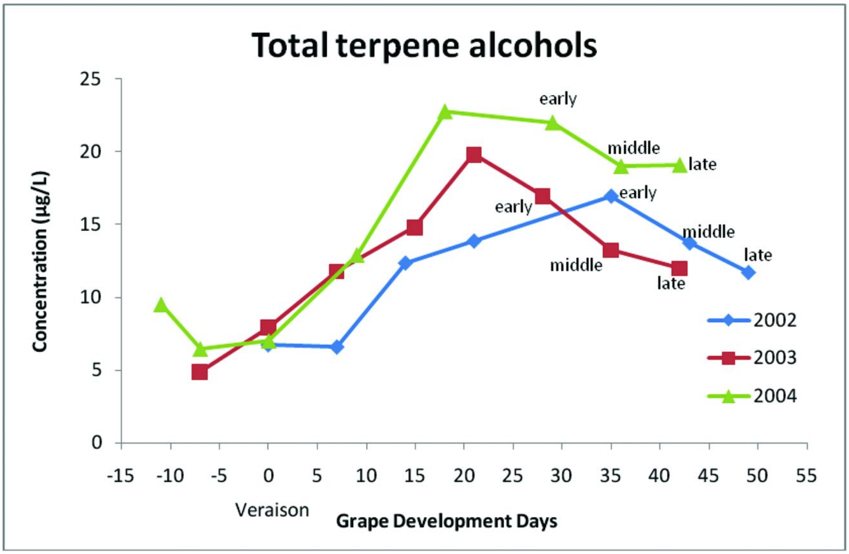

Figure 4. Development of total terpene alcohols during grape development.

Large amounts of benzyl alcohol, phenylethyl alcohol and phenol were found in grape juices. As the grapes developed, their concentration dramatically increased. These compounds contribute to the typical floral aroma in grapes and wines, especially phenylethyl alcohol. However, phenylethyl alcohol in wine is largely formed by fermentation yeasts through shikimate pathway (*31*), thus there is no direct correlation between the amount present in grapes and that in wines.

1-Octen-3-ol, having a remarkable mushroom-like odor, has been reported to be present in many wines (*32*, *33*). It has been reported that 1-octen-3-ol is formed during grape ripening as a result of gray mold attack, it is a defect if 1-octen-3-ol is present at a high concentration (*34*). The concentration of 1-octen-3-ol did not change during the grape growing season.

Small amounts of vanillin and γ-nonalactone were found in the grapes. Vanillin decreased during grape maturity, though a slight increase was observed in the very early stage, while γ-nonalactone didn't change along with the grape development. Like monoterpenes as well as norisoprenoids, these compounds occur in grapes and wines predominately as glycosidically bound precursors, and arise from the enzymatic hydrolysis and acid cleavage during the wine making process (*2*). To investigate their effect on wines, it is necessary to study their glycoside precursors. Other volatile compounds were also quantified in our study, such as long carbon chain aldehydes, and methyl and ethyl vanillates. There were no obvious trends for these compounds, though they may contribute to grape and wine aroma.

Big variations in concentration for some compounds have been associated with vintages. Year 2003 and year 2004 were much hotter than year 2002, and the total heat accumulation during the grape growing season could have contributed to the variations. It has been documented that climate affects the volatile composition in the grapes. Herrick and Nagel (*35*) found the phenol content of Riesling wines from Alsace (13 mg/L) was much lower than those from eastern Washington State and California (123 mg/L). Ewart et al. (*36*) compared different vineyard sites in south Australia and found that total volatile terpenes in the grapes increased more slowly in the cool site but were at higher concentrations than in the warm site. However, the difference among different vintages was not clear enough to make any conclusion from this study.

In summary, analysis of grape volatiles during three growing seasons showed that different compounds undergo different routes during grape development. Moreover, since glycoside precursors in grapes are important to wine aroma, hydrolysis studies should be done to better understand the correlation between grape composition and wine quality.

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Chapter 7

Accumulation of C_{13}-Norisoprenoids and Other Aroma Volatiles in Glycoconjugate Form During the Development of Pinot Noir Grapes

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The development of bound aroma potential compounds in Pinot noir grape juice was investigated using stir bar sorptive extraction-gas chromatography-mass spectrometry (SBSE-GC-MS) after enzymatic and acidic hydrolysis. The Pinot noir grape berry samples were collected during the growing seasons of 2002, 2003, and 2004. The results showed that the amount of C_{13}-norisoprenoids released from bound precursors was more than ten times the amount of the free form in juices, and these compounds dramatically increased during grape maturation. Vanillin and γ-nonalactone also showed dramatic increase during grape maturation. Benzenoid compounds (benzyl alcohol and phenylethyl alcohol) decreased in the very early stage, and increased during later stages of maturation. However, bound monoterpenes did not increase at the later stage of maturation.

Keywords: Glycoside precursor; aroma-active compounds; Pinot noir grape; stir bar sorptive extraction (SBSE)

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Introduction

Quality wines have different flavor properties, which often depend on varietal characteristics. Their typical flavor is mainly due to aroma compounds that are present in the grapes, whether they are in free volatile form or in bound form (*1*). Therefore, the grape aroma in both free and bound form is critical to both the grape grower and the wine maker.

During ripening, grape berry quality generally reaches a peak and then declines as fruit becomes overripe. It is at this peak, or optimum stage, of maturity that winemakers aim to harvest the fruit. However, it is still a challenge to determine precisely when the optimum is reached. Although the final judgment is the subjective assessment of tasting the end product, this is too late, too slow and costly. Tasting fruit to evaluate maturity may work only within very confined limits, mostly because sweetness, acidity, and astringency can be tasted, while many flavor and aroma compounds are locked up as non-volatile glycosides and are only released during the winemaking process (*2*).

A preliminary sensory evaluation of wines from grapes harvest at one week early, commercial, one week late maturity showed that wines from the late harvest grapes had more complex aroma with more floral, more dried fruit and more oak-like aroma, while the wines from early stage grapes showed the highest fresh fruity aroma (unpublished data). Instrumental analysis has shown that grape maturity (harvest date) significantly affects some key aroma compounds in wine (*3*). However, the relationship between grape development and wine aroma is still unclear.

The development of free form aroma compounds in Oregon Pinot noir were investigated by stir bar sorptive extraction- gas chromatography-mass spectrometry (SBSE-GC-MS) (*4*). It was found that the C6 alcohols continuously decreased during the berry development whereas the C6 aldehyde only began to decrease after reaching the harvest maturity. Benzyl alcohol and 2-phenylethyl alcohol dramatically increased over the whole growing season. However, most of the free monoterpene alcohols (geraniol, nerol and citronellol) as well as β-ionone only accumulated at the early grape development stage, and their concentration did not show significant increase at the late stage of ripening. It is hypothesized that these compounds were accumulated as glycosides or other bound precursors.

In the current experiment, the bound aroma potential compounds were released from their precursors in grape juice by enzyme hydrolysis under acidic condition. These hydrolyzed aroma compounds were then quantified by SBSE-GC-MS. Our objective is to investigate the development of bound aroma compounds during grape development, which will further help to understand the relationship between grape maturity and wine aroma.

Materials and Methods

Chemicals

All volatile standards and internal standards were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide was bought from J.T.Baker

(Philipsburg, NJ), citric acid was from Staley Manufacturing Company, (Decatur, IL), and sodium chloride was from VWR International (West Chester, PA). The MACER8TM FJ enzyme solution, which contained a balanced mix of pectinases and pectin lyase, and other enzymatic activities, was provided by Biocatalysts Limited (Wales, UK)..

Preparation of Standard and Internal Solutions

Citric acid buffer solution (0.2 M) was prepared by dissolving 42g of citric acid into 1 L of Milli-Q water (Continental Water System, Millipore Corporation, Billerica, MA), and then adjusted the pH value to 3.1 using diluted sodium hydroxide solution. Standard stock solutions (about 1000 ppm) were prepared in ethanol individually and stored at -15°C. Before analysis, the standard stock solutions were diluted to the proper concentrations of working standards in the citric buffer solution. An internal standard solution was made by dissolving 1.93 ppm of octyl propanoate, 0.55 ppm of *trans*-carveol, and 0.94 ppm *trans*-2-nonenal in ethanol, and was stored at -15°C.

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Grape Sampling and Juice Preparation

Pinot noir grapes were grown at the Oregon State University experimental vineyard located in Alpine, OR. During the growing seasons of 2002, 2003 and 2004, ten clusters from different vines were randomly picked in the vineyard at different development stages and were immediately frozen at -29°C as described previously (*3*). Berries were destemmed while still frozen, and then placed in a glass jar and kept at -23°C. Prior to analysis, about 200g of grape berries were thawed at 4°C overnight and then ground using a commercial blender (Waring Products Division, New Hartford, CT). After settling for 5 min, skins and seeds were separated from the juice using cheese cloth, and then the grape juice was immediately analyzed.

Bound Volatile Isolation and Hydrolysis

Glycosides in the grape juice were obtained using a C18 SPE column (J.T.Baker, Philipsburg, NJ) as described in literature with minor modification (*5*). Each C18 SPE column was pre-conditioned with 10 mL of methanol, then with 10 mL of Milli-Q water. Five mL of grape juices was slowly loaded on the C18 column. After the sample loading, the SPE column was washed with 10 mL of Mili-Q water and then with 6 mL of pentane/dichloromethane (2:1, v/v). The glycosides were finally eluted from the column with 6 mL of methanol into a 40 mL of vial. The methanol eluent was concentrated to dryness at 45°C under vacuum. Twenty ml of 0.2 M citrate buffer solution and 100 µl of Macer8™ FJ enzyme solution (Biocatalysts Limited) were added into the glycoside extracts. The mixture was incubated at 45°C for 24 hours for enzyme hydrolysis at pH 3.1. The hydrolyzed solution was cooled to room temperature, and immediately analyzed.

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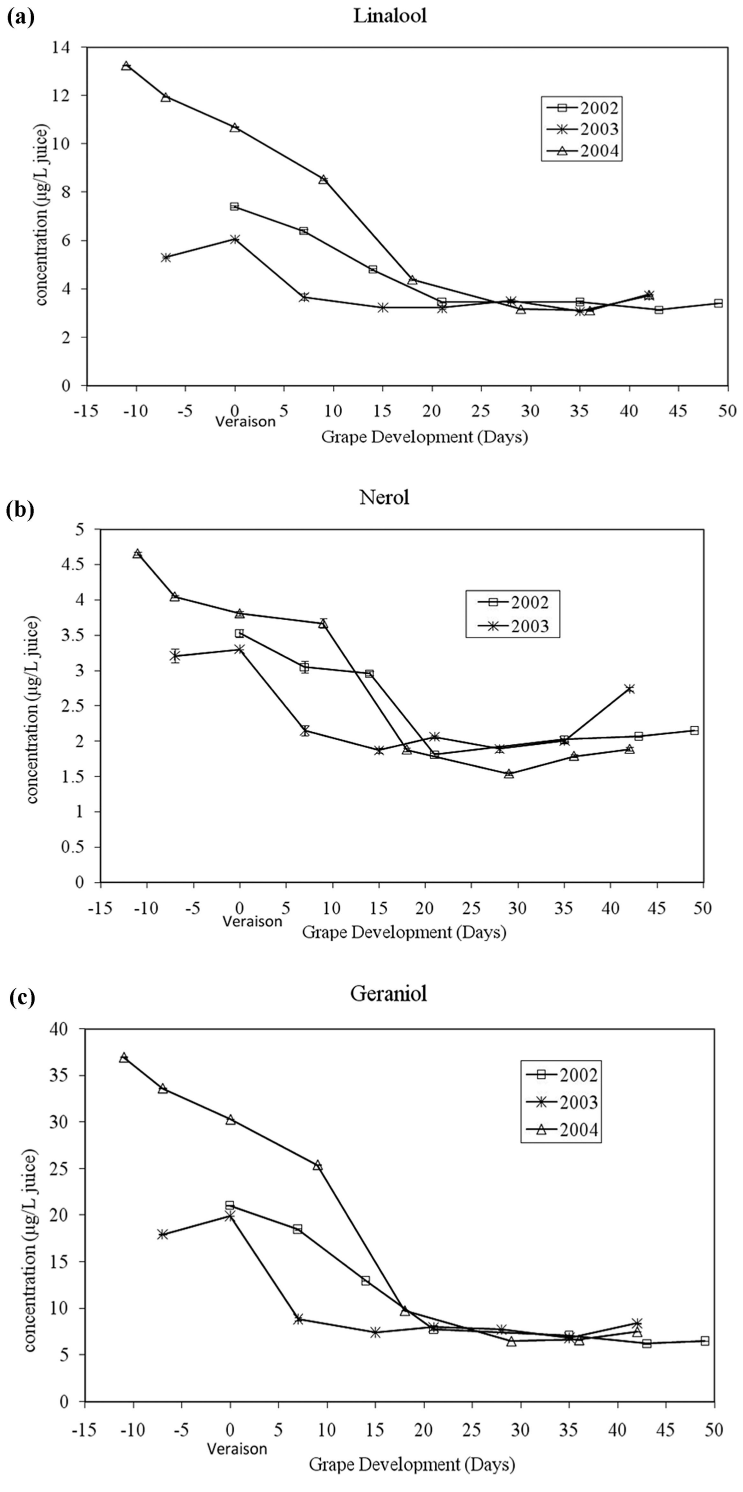

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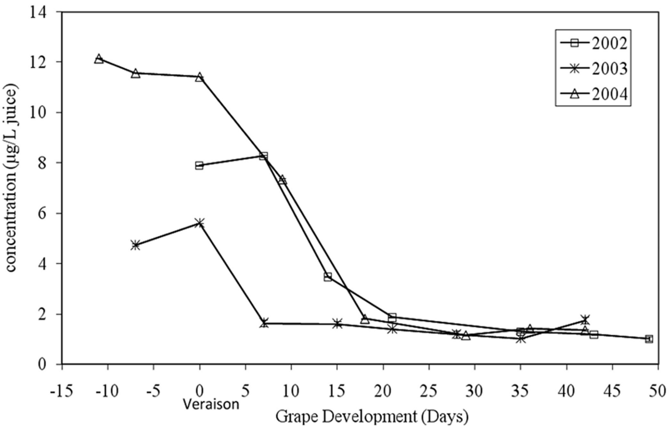
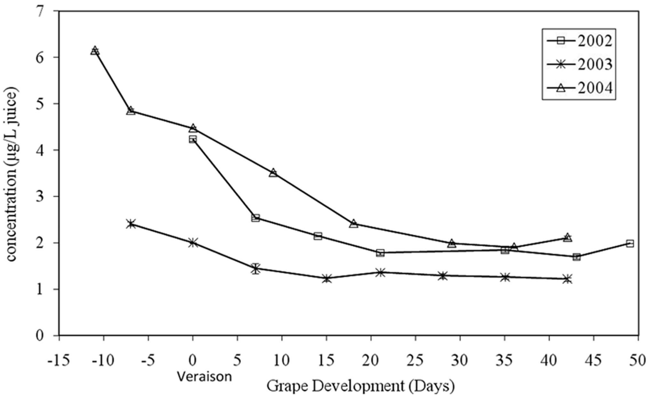
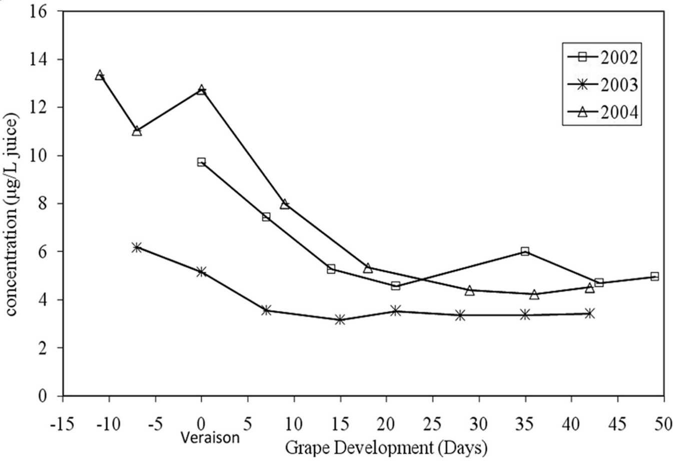
(d) Citronellol



(e) Linalool oxide



(f) α-Terpineol



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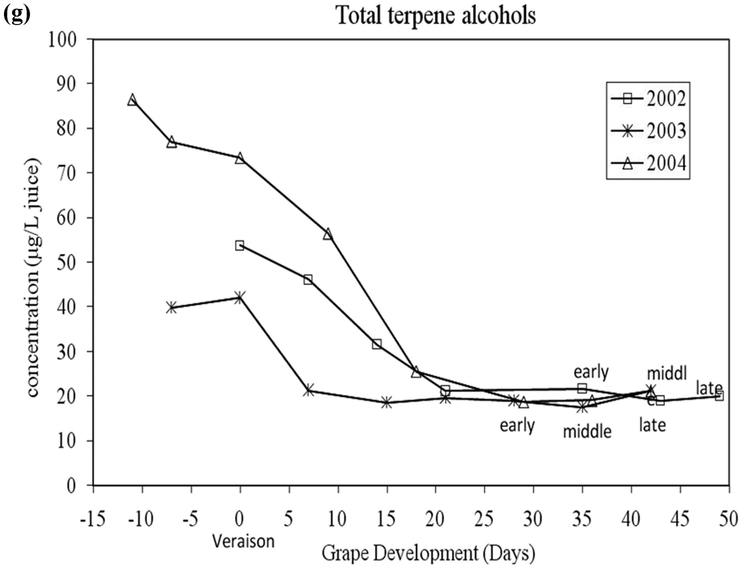

Figure 1. (a−g) The development of bounded monoterpenes in Pinot noir grapes during 2002, 2003, and 2004.

Aglycone Analysis by SBSE-GC-MS

The released aglycones were analyzed by SBSE-GC-MS described previously (*4*). Ten mL hydrolyzed solution and 10 mL of 0.2 M citrate buffer (pH=3.1) as well as 6 g of sodium chloride were mixed into a 40 mL vial, and 20 µL of internal standard solution was also added. A twister bar coated with PDMS phase (1 cm × 100 mm, Gerstel Inc., Baltimore, MD) was used to extract the volatile compounds from the solution. The twister bar was constantly stirred for 3 hours at a speed of 1000 rpm. After extraction, the twister bar was rinsed with Milli-Q water, dried with tissue paper, and used for GC-MS analysis.

The analytes were thermally desorbed in the TDU in splitless mode, ramping from 35°C to 300°C at a rate of 700°C/min, and held at the final temperature for 3 min. The desorbed analytes were cryofocused (-80°C) in a programmed temperature vaporizing (PTV) injector (CIS 4, Gerstel Inc.) with liquid nitrogen. The solvent vent injection mode was employed with a venting flow of 25 mL/min and a venting pressure of 10 psi for 0.01min. After the desorption, the PTV was heated from -60°C to 250°C at a rate of 10°C/s and kept at 250°C. A ZB-FFAP capillary GC column (30m, 0.32mm ID, 0.25µm film thickness, Phenomenex, Torrance, CA) was employed to separate the analytes. The column carrier gas was helium at 2 mL/min. The oven temperature was programmed initially at 40°C for 2 min, then increased at 6°C/min to 180°C, further increased at 4°C/min to 240°C, and held at the final temperature for 20 min.

Standard Calibration and Aglycone Quantification

The released volatile compounds were quantified using a standard curve of the specific compound as described previously (*3*). The standard stock solutions were prepared by dissolving around 10 mg/L each compound individually in ethanol solution. Before analysis, stock solutions were added into citrate buffer solution to make the mixed standard solution and then was diluted with synthetic wine to give a range of calibration concentrations. Six g of sodium chloride and 20 µL of internal standard solution was added, and the compounds were extracted with stir bar for 3 hours, and analyzed using the same procedure as described previously. The MS total ion data (scan 35 to 300) was collected but selective ion monitoring was used for quantification. Triplicate analysis was performed on all samples, and the average values were reported.

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Results and Discussion

Most grape aroma compounds are present in the grape either as free volatiles, which may contribute directly to odor, or as non-volatile bound sugar conjugates. The bound sugar conjugates, or glycosides, are nonvolatile and, for the most part, represent aroma potential. The bound precursors can undergo acid or enzyme hydrolysis, releasing free volatiles and potentially enhancing aroma (*6*). Research by Francis and co-workers compared the effect of hydrolysis conditions on the aroma compounds released from grape glycosides (*7*, *8*). Based on sensory descriptive analysis, they found that hydrolysis catalyzed by only enzymes had no detectable effect on aroma, whereas acid-catalyzed hydrolysis produced sensory properties similar to those of bottle aged wines. Therefore, in this study, enzyme combined with mild acid (pH=3.1) hydrolysis was investigated as a way to release the bound aroma compounds from glycoside extracts in grape juice.

Grape glycoside precursors generally released a wide range of compounds, which represent, in part, the potential aroma of a grape variety. These compounds include monoterpene alcohols, C_{13}-norisoprenoids and other minor compounds, which were the main target compounds in this experiment. The quantification method for these target compounds in grape juice was previously developed using SBSE-GC-MS for the analysis of free form aroma compounds (*4*). Since the same citrate buffer solution was used in this experiment as that for making the calibration curves, the method is also suitable to quantify compounds in the hydrolyzed solution.

Monoterpenes belong to the secondary plant constituents, of which the biosynthesis begins with acetyl-coenzyme A (CoA) (*9*). They are largely present in the skin of the grapes, and glycoside precursors are the most abundant form (*10*), which varies with different varieties of grapes (*11*).

Enzymatic hydrolysis of terpene alcohol glycoside releases free terpene alcohols to the wine. This hydrolysis involves two steps (*10*). In the first step, a α-L-rhamnosidase and a α-L-arabinofuranosidase or a β-apiofuranosidase (depending on the structure of the aglycone moiety) cleave 1,6-glycosidic linkages to give monoterpenyl β-D-glucosides. In the following step, β-glucosidase hydrolyzes the monoterpenyl β-D-glucosides to give monoterpene alcohols (*12*).

Grapes have β-glucosidase activity but very low α-rhamnosidae, a-arobinosisade or β-apiosidase activities. In addition, grape β-glucosidase has very low activity at acidic pH (*13*). Wine yeasts have some β-glucosidase activities, but depending upon the origin of the yeasts, the β-glucosidase activities can be either inhibited by sugar or alcohol (*14–16*). The glycoside concentration and composition of grape must can also induce some yeast strains to generate β-glucosidase activites. There is evidence that non-*Saccharomyces* yeast can contribute significantly to a wine's final flavor and aroma (*17–23*), because many non-*Saccharomyces* yeasts can produce significantly higher amounts of β-glucosidase than S. cerevisiae (*24–26*).

Besides enzymatic hydrolysis, acidic hydrolysis can also release the monoterpene alcohols from their precursors. It has been confirmed that the progressive release of aroma with long periods of mild acid hydrolysis is reflected in the increase in intensity of the aroma attributes in wines undergoing natural aging (*8*).

Among all of the terpene alcohols analyzed, geraniol is the most abundant monoterpene alcohol released from hydrolysis, with 10 μg/L juice. Other important terpene alcohols include linalool, a-terpineol and nerol.

Figure 1 shows the changes of concentration of six monoterpenes, as well as total terpene alcohols during grape development across three seasons. All monoterpenes decreased in the early stage of grape development, one reason may be that the results are presented as the concentration in grape juice, rather than the concentration in grape berries. Berry growth is mostly due to water increase, where the juice yield before veraison is much lower than that close to harvest. Therefore, in the early stages of ripening, berry volume increases quickly so that aroma precursors in juices become diluted. In the later stage, berry volume remains constant, and the aroma precursor concentrations begin to increase. However, it is surprise to see that the terpene alcohol glycosides only increased slightly, depending on the vintage, or did not increase at all. This result disagreed with previously studies (*27*, *28*), possibly due to the fact that Pinot noir is a neutral variety, and the contents of terpene alcohol content is low.

Monoterpene alcohols can undergo rearrangement under acidic conditions during vinification and maturation (*29*). For example, linalool can be transformed in an aqueous acid medium to α-terpineol by cyclization, to hydroxyl-linalool through hydration in the seventh position and to geraniol and nerol by a nucleophilic 1,3-transition. Since the transformation is very complicated, the total terpene alcohols should be considered.

C_{13}-norisoprenoids are considered to be very important to red wine aroma. The most important C_{13}-norisoprenoids in wine are β-damascenone and β-ionone, imparting 'rose' or 'exotic fruit' and 'violet' or 'raspberry' odor, respectively (*7*, *30*, *31*). 3-Hydroxy-β-damascenone was also considered as an important bound aroma compound in grapes (*32*). However, this compound has not been reported in wines, suggesting it is converted to β-damascenone or other compounds during winemaking.

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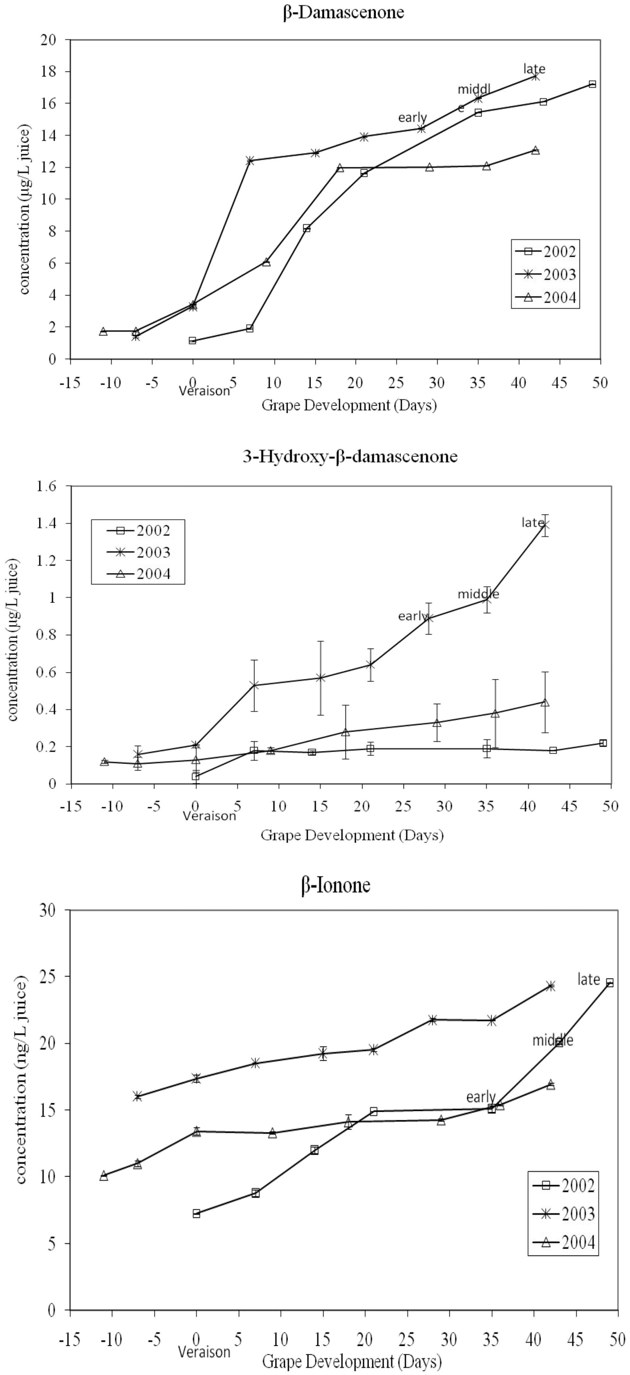

Figure 2. The development of bounded C_{13}-norisoprenoids in Pinot noir grapes during 2002, 2003, and 2004.

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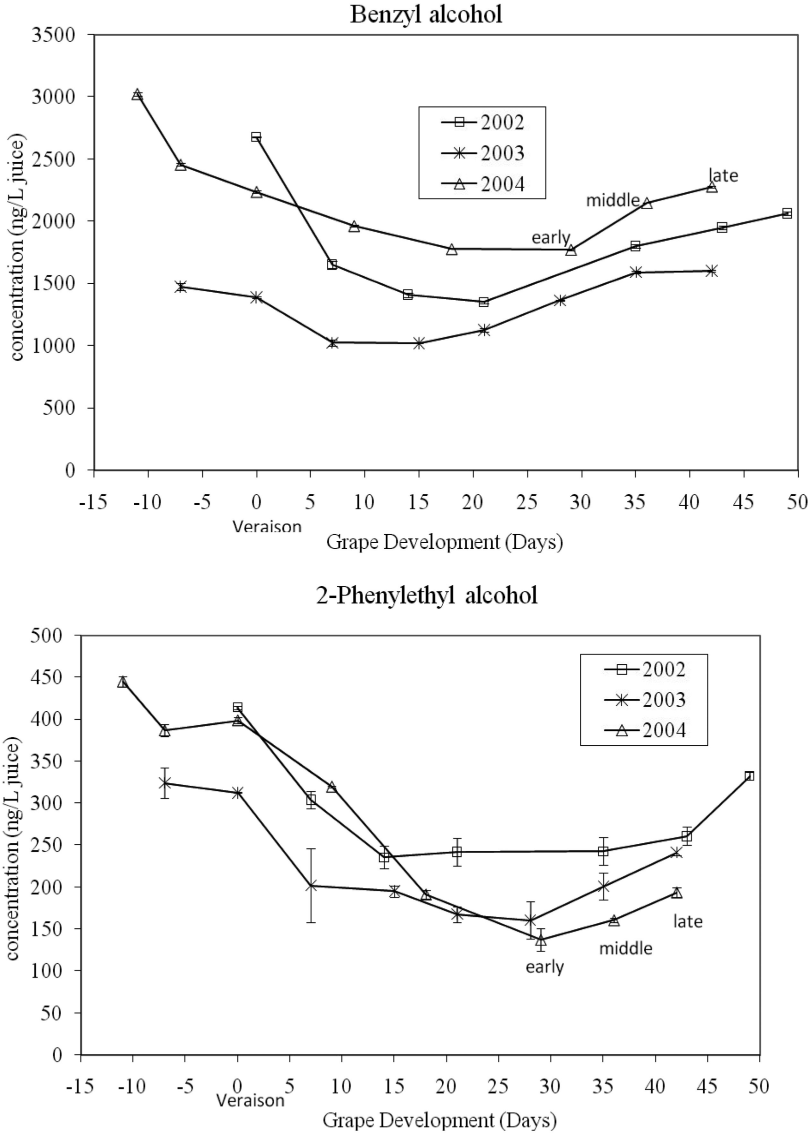

Figure 3. The development of phenylethyl alcohol and benzyl alcohol in Pinot noir grapes during 2002, 2003, and 2004.

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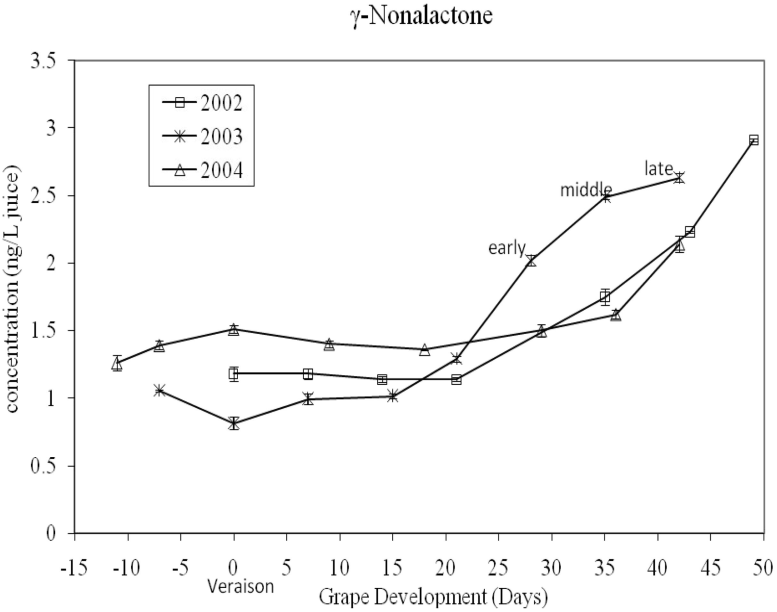

Figure 4. The development of γ-nonalactone in Pinot noir grapes during 2002, 2003, and 2004.

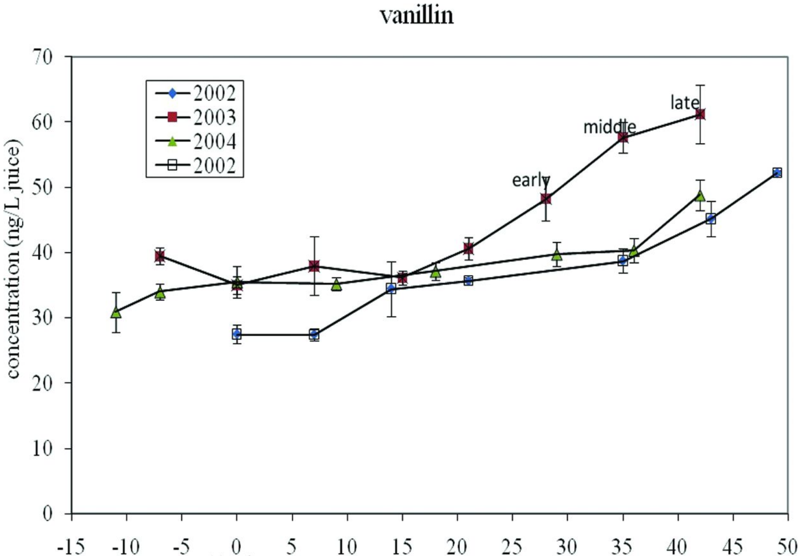

Figure 5. The development of vanillin in Pinot noir grapes during 2002, 2003, and 2004.

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Figure 2 shows the development of bound β-damascenone, β-ionone, and 3-hydroxy-β-damascenone during 2002, 2003, and 2004. Compared to free form (*4*), about 10~100 times more β-damascenone was hydrolyzed from bound precursors in the same grape juices, and its amount was dramatically increased along with grape development, even close to harvest time. These results are consistent with the literature (*33*). C_{13}-norisoprenoids are generated through the breakdown of carotenoids. Throughout the grape development, carotenoids are accumulated early on in berry development to protect berry tissues from oxidative stress, and it appears that the carotenoids are converted to C_{13}-norisoprenoids after veraison. Similar trends were also found for β-ionone and 3-hydroxy-β-damascenone. However, the concentration of bound β-ionone is less than its free form in juice. This indicated that unlike other C_{13}-norisoprenoids, the glycoside precursor of β-ionone is not the major form present in grapes.

In our previous study, it was found that wines made with late stage grapes contained more β-damascenone and β-ionone than those made with the early stage ones (*3*). Our results confirmed that this difference is mainly dependent on the increase of bound aroma precursors during grape maturation. Therefore, late harvested Pinot noir grapes could produce wine with fruitier, more berry-like aroma, which is associated with these compounds.

Phenylethyl alcohol decreased in the very early stage of grape growing, and then increased in the late stage (Figure 3). However, this compound can be generated from yeast during fermentation (*34*), so its concentration in grapes cannot be easily related to its concentration in the final wine. Similar trends were also found for benzyl alcohol (Figure 3).

A strong increase trend with grape maturity was observed for γ-nonalactone, and this increase was only profound when grapes approached maturity (Figure 4).

Although vanillin can be extracted from oak barrel during barrel aging (*35*), it can also originated from grape. The results showed that the bound vanillin increased during the grape development (Figure 5).

In conclusion, the bound aroma compounds in Pinot noir grapes were highly related to grape maturity. The contents of bound β-damascenone and β-ionone increased rapidly after veraison and extended through ripening; benzyl alcohol and phenylethyl alcohol decreased before veraison and increased when approaching maturity, γ-nonalactone only increased when approaching maturity. The data supported previous study that the wines made from those later maturity grapes have higher concentration of these compounds, and exhibit stronger fruity, berry aroma. However, the terpene alcohol glycosides had very little increase at later maturity.

Acknowledgments

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Chapter 8

Evaluation of the Impact of an Archaic Protocol on White Wine Free Aroma Compounds

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Traditional Georgian wines are produced using long-time skin contact, often in jars of clay in absence of sulfur dioxide and other adjuvants.

Long-time skin maceration white wines are recently produced at industrial level also in Italy to extract more antioxidant phenolic compounds and obtain a possible different taste and varietal flavor development by aging in quite stable conditions. The consumers, even if rather skeptic for the sensory wine character, judge with an emotional appeal these 'interesting' wines.

The present research involves mostly wines produced from the Garganega grape variety, which is spread in the Veneto region in northern Italy and it is used to obtain the renowned Soave white wine.

Applying SPE and HS-SPME GC-MS analyses on the free fraction of wine as such we investigated the free aroma variation and compared the products from the typical free-run vinification with those obtained via a skin maceration of several months.

Particular attention has been paid to the prefermentative and varietal compound as C6-alcohols, monoterpenols, benzenoids and norisprenoids, as so as to fermentation derived substances like hydroxyalkyl ethyl esters, lactones, amides, furanols, aryl

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alcohols like tyrosol and tryptophol, HCA derived ethyl esters, some sulfur compounds, etc.

An important increment of some of these compounds has been already observed in long-time skin fermented wines and some of this variations can be connected to sensorial nuances in agreement with what is reported in the literature.

All the wines have been submitted to descriptive profiling and preference tests.

Finally, this ‘ancient’ vinification method could offer an interesting chance – depending on the variety characteristics – for preparing ‘unusual’, properly aged products.

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Skin contact is a crucial step in winemaking; in red winemaking it assures a higher content in phenolic compounds so that a better colour stability (*1*) and wine structure is conferred to the wine, while in white wine it improves the extraction of aroma precursors which mainly present in the skin (*2–4*).

In white wine this particular stage of the winemaking can also have unpleasant effects such as the development of herbaceous aroma or bitter flavours. Therefore, the maceration in white winemaking must be optimised carefully. Nowadays, most of the common white winemaking protocols involve a very short (up to 1 day) pre-fermentative skin-contact at low temperature (*4*, *5*) followed by the yeast inoculation and the regular alcoholic fermentation with no skin contact occurring.

Back in the days when the wine was firstly produced, the winemaking procedures applied for the wine production were quite different. Georgia is deemed to be the oldest wine producing region in the world; the wine was produced by burying the grape juice together with the grape skin in clay jar underground through the winter (*6*). In these days, such archaic procedure is still applied, producing interesting wines with peculiar aromatic characteristics.

The effects of very long maceration contact, such as the one performed in Georgia, on Italian white wines was never taken into account. In particular a very important class of molecules, such as the fermentative sulfur compounds, was never considered. The biogenesis of these molecules seemed related to the availability of amino-acidic precursors. It is known that skin contact can enhance the level of several amino acids (*1*) and this could be potentially related to sulfur compound biosyntheses.

In this paper, the effect of this archaic procedure on a vast range of volatile and non-volatile species and on the sensory characteristics was investigated. Two important Italian varieties e.g. Vitis vinifera Cv. Garganega (Verona) and Vitis vinifera Cv. Verdicchio (Ancona) were investigated.

Finally the free forms of several classes of fermentative and varietal aroma compounds were taken into account and a significant number of fermentative sulfur compounds was investigated for the first time.

Aroma descriptors as tobacco, chocolate and honey that appeared to be strongly related to skin maceration (*7*) were found in agreement with the increment of specific molecules observed in the long-maceration experiments preformed.

The fermentative sulfur compounds appeared affected by the skin-contact length, supporting the theory that the higher availability of amino acids could impact on the production of these species.

Experimental Section

All the wines were produced at industrial scale in stainless steel tanks. Healthy grape (ca. 21° Brix) was processed in a local winery. The grape was split into two batches. The first one was submitted to traditional white winemaking (WW). Sodium metabisulfite (50 mg/L) was added to the grape before pressing. The grape was then pressed and cleared of the skins and stems. The second batch was treated according to a red winemaking (MW): the grape was submitted to soft pressing and then the juice was kept with the skins for 4 months. The two batches were inoculated with the same Saccharomyces cerevisiae strain (detail). The temperature for both batches was kept at 17°C for the whole fermentation.

The batch processed according to the long skin contact protocol was kept without any addition of SO_2 for the first 4 months. Both the batches spontaneously undergo malolactic fermentation.

After 4 months all the wines were added of 60 mg/L of SO_2, bottled and submitted to analysis. Three separated vinifications were performed for each wine (i.e. Verdicchio and Garganega) ad for each experiment (traditional winemaking and long skin contact winmaking).

Solid Phase Extraction (SPE), using 1g ENV+ cartridges (Isolute, IST Ltd., Mid Glamorgan, UK), was performed to quantitatively extract the aroma compounds; subsequently the extract was injected into a gas chromatography – mass spectrometry (GC-MS). The SPE method was performed with an Aspec XL Sample Processor (Gilson Inc., Middleton, USA). The cartridges were sequentially conditioned with methanol (10 mL) and MilliQ water (10 mL). A total of 76 mL of wine sample diluted 1:4 v/v, with distilled water and added with 1-heptanol as internal standard (500 µg/L), were loaded onto the cartridge. The cartridge was then rinsed with 10 mL of distilled water. The residual were washed with 10 mL of distilled water. The free aroma compounds were eluted with 9 mL of dichloromethane. The solution was dried with Na_2SO_4 and concentrated to 0.4 mL by a gentle nitrogen flow (*8*, *9*).

GC-MS analysis was performed with a 6890N Network GC System coupled with a 5978B inert XL EI/CI MS (Agilent Technologies, Milano, Italy), equipped with a HP-WAX Bonded PEG fused silica capillary column (60m x 320 µm i.d. x 0.25 µm filn thickness; Agilent Technologies). MS conditions were: electron impact energy 70 eV and MS source temperature 230°C. GC injector temperature was 250 °C and helium was used as carrier (flow: 1.5 mL/min). Column temperature program was: 50 °C (4 min), 4 °C/min to 240 °C, 240 °C (16 min).

Fermentative sulfur compounds (i.e. molecules originated from the yeast metabolism during the wine fermentation) were analyzed according to a previously published method (*10*). The choice of the best fibre to study the quoted fermentative sulfur compounds was made according to our previous experiences and to literature data (*8–11*). The fibre chosen was a

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carboxen-polydimethylsiloxane-divinylbenzene (CAR-PDMS-DVB; 50/30mm, 2 cm long). The sampling was carried out with the MPS2 Autosampler (Gerstel GmbH & Co.KG, Mülheim an der Ruhr, Germany). The SPME holder for automated sampling and the fibres were purchased from Supelco (Bellefonte, PA, USA). The fibres were conditioned before the use according to the producer's instructions. The sample (5 mL) was transferred into a 20 mL vial, and 2 g of NaCl was added. HS-SPME sampling was carried out at 35°C for 30 min.

GC-MS apparatus was a GC 6890N (Agilent Technologies) equipped with a DB-Wax capillary column (60 m x 320 μm ID x 0.25 mm film thickness, Agilent Technologies) and coupled with a MS 5975B mass spectrometer (Agilent Technologies). Gas chromatography conditions were: GC injector temperature 250°C, injection in splitless mode for 1 min, oven temperature program: 35°C (5 min), 1°C/min to 40°C, 10°C/min to 250°C. Helium was used a carrier gas (flow 1.5 mL/min).

The chromatographic analyses were carried out in single ion recording (SIR) mode. Identification of the analytes and internal standards was achieved by co-injecting the pure reference compounds and using the NIST library; mass fragments adopted for the quantification are according to Fedrizzi et al. (*8*, *10*).

A calibration curve for each analyte was prepared according to the internal standard method. Validation was performed on a dry red wine (13% alcohol strength v/v) treated twice with charcoal (3 g/L) to remove any sulfur compounds detectable by the proposed HS-SPME/GC-MS method as reported elsewhere (*10*). Linearity and sensibility were verified in the concentration ranges typical of red wines. Calibration curves were prepared using 7 concentration levels and 5 replicate solutions per level; detection limit (L_D) was calculated (Table 1) according to Hubaux-Vos procedure (*12*).

The data were statistically evaluated and plotted using STATISTICA v7.1 (Statsoft Italia S.r.l., Padova, Italy).

Results and Discussion

The analysis of the volatile fraction of these wines provided some interesting information. In particular some results were in agreement with what is the theory behind aroma compounds evolution, while other appeared to be new. This was the first time the whole range of fermentative and varietal aromas, in their free components, was submitted to thorough analysis for this particular winemaking approach.

As reported in Table 1, fermentative esters seemed negatively influenced by long skin-contact. This would be in agreement with the idea that "dirty" fermentation can lead to a lower amount of esters produced (*13*).

Monoterpenols (Table 2) did not appear to be significantly affected by maceration techniques.

C13-Norisoprenoids (Table2) were not influenced by long maceration except for the 3-oxo-α-ionol, which increased markedly in the long skin contact wines. This molecule has been described as having a honey/tobacco note and it seemed

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correlated with the skin contact (*14*). The positive correlation with the extended skin contact would enforce this theory.

Four lactones were quantified in this experiment (Table 3); except for the γ-butyrrolactone, all increased in macerated wines. These molecules can have a strong impact in sweet wines and Botrytis wines, imparting "apricot" and "peach" notes (*15*, *16*). Nonetheless, none of these descriptors was found in our sensory analysis.

Finally, several benzenoids were quantified. 2-Phenylethanol, benzyl alcohol and benzaldehyde changed considerably in macerated wines. The first two probably changed because of effect of grape skin solids on east meatabolism, while in the latter case the causes remain still unclear. A putative pathway would involve the oxidation of benzyl alcohol, but in normal winemaking condition this does not happen. Such mechanism seem to occur in anaerobic condition (*17*, *18*), even though it is not proved to be our case.

Particularly interesting is the analysis of the methyl salicylate; this molecule has a floral/chestnut honey sensory note and is present in tea leaves mainly as a β-glucoside and β-primeveroside (*19*, *20*). In Vitis vinfera this it is a varietal marker for the Garganega and Verdicchio grape varieties and it is present as different β-diglicosides (*21*). In this two grape varieties methyl salicylate is present in both free and bound forms in concentrations much higher than in other varieties. Analysis of the trifluoroacetylated grape glycosidic fraction demonstrated that methyl salicylated is present in Verdicchio grape as β-glucoside and β-primeveroside but also in other β-diglycosidic forms (data not shown).

Other benzenoids, such as aceto-, propio- and butyrrovanillone, and aceto-, propio- and butyrrosyringone were reported to be strongly correlated to skin contact (*7*); our experiment confirmed such evidence, strengthening the hypothesis of a crucial role of these molecules in defining the aroma profile of wine produced with extended skin-contact.

A wide range of fermentative sulfur compounds was investigated (Table 4). These compounds are originated from amino acidic precursors by the yeast metabolism (*22*, *23*) though for many of them a clear understanding on their formation is not available as yet.

Fermentative sulfur compounds are deemed to impart a range of different sensory contribution to wine aroma (*23–28*); in the past they were mainly investigated for their negative influence but now a new interest arisen for the potentially positive impact on wine typicality and complexity.

In the present work several fermentative sulfur compounds, belonging to different chemical classes were investigated. In particular, they were chosen among the most important and highly impacting on wine quality.

All the sulfur compounds showed an increment in the macerated wines. This evidence would support the idea that these molecules are originated from amino acidic precursors which are released from the skin during the prolonged skin-contact. Must turbidity could also significantly impact the production od sulfur compounds as already highlited by Karagiannis & Lanardis (*29*).

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Table 1. Long skin contact effect on the level of several fermentative esters. Data are expressed in µg/L. SD: standard deviation

	Garganega								*Verdicchio*			
	White Wine		*Macerated Wine*		*White Wine*		*Macerated Wine*		*White Wine*		*Macerated Wine*	
	2008				*2009*				*2008*			
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Hexyl acetate	79.2	16.6	3.8	2.8	51.4	2.3	7.1	7.0	99.5	21.1	20.8	2.9
Isoamyl acetate	2378.9	978.6	182.5	47.7	1375.4	253.3	278.9	16.1	1232.9	368.3	572.8	40.3
β-phenylethyl acetate	468.4	383.4	23.5	5.8	171.7	26.4	28.0	2.3	201.4	59.3	54.7	11.7
Ethyl butyrate	256.5	164.7	117.4	40.5	210.7	18.0	131.4	4.9	299.0	51.1	298.9	12.9
Ethyl hexanoate	754.8	258.6	302.8	69.7	651.8	100.8	315.7	13.2	672.7	224.7	569.2	37.7
Ethyl octanoate	1334.6	434.3	305.7	87.6	1103.7	253.1	413.0	28.6	1100.6	304.4	649.4	112.9
Ethyl decanoate	498.5	167.0	90.9	19.6	494.2	99.4	90.7	18.5	240.4	56.7	144.9	36.4
Ethyl 3-idroxybutyrate	223.8	57.5	232.6	21.5	259.5	78.3	340.9	17.6	331.1	64.1	294.7	10.8
Ethyl 4-idroxybutyrate	2084.2	416.5	4157.8	544.9	564.7	86.1	3408.1	223.0	1244.0	425.4	2084.6	183.5

DMS, which is thought to be originated from the S-methyl methionine (*30*, *31*), significantly increases during skin contact. Similar behaviour is shown by DES, even though no precursors have been identified for this sulfide.

The increment of disulfides appeared smaller and below their sensory threshold anyhow.

Finally, thioalcohols concentration (i.e. MTE, MTP and MTB) doubled in all the long skin-contact experiments. These compounds are thought to be originating from the relevant amino acid via Strecker mechanism, even though the only one demonstrated as yet, is the MTP mechanism from methionine.

According to the UNI 10957:2003 norm and to the international standards ISO 8586-1:1993 and ISO 8586-2:1994, 11 trained panellists were selected (www.iso.com). The tasting order has been randomised for each taster, which was operating in separated booth and using dark glasses to avoid any bias. The definition of the sensory descriptors was achieved according to the ISO 11035:1994 norm. The sensory profile of the white wines produced appeared to be obviously influenced by the skin-contact (Figure 1). As expected, macerated wines showed a higher astringency and bitterness, but according to the panellists this was not runing the overall wine quality.

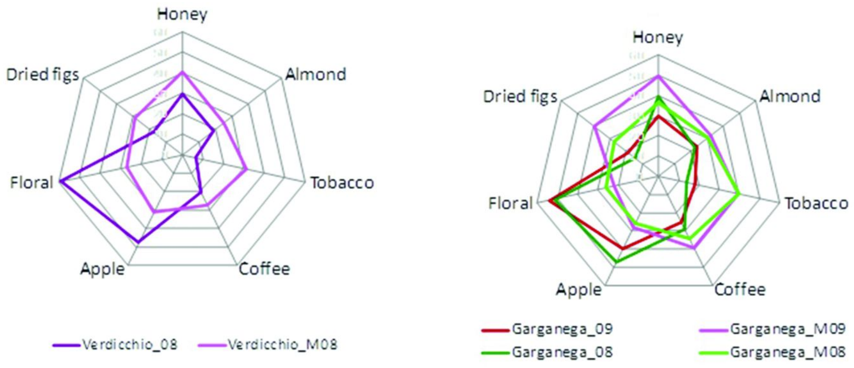

Figure 1. Sensory evaluation map of the Verdicchio and Garganega regular and macerated wines.

Aroma descriptors such as honey, dried figs and tobacco resulted to be strongly enhanced by the extended skin contact. In a pervious work, Leigh and co-workers (*7*) reported that honey and tobacco descriptors were positively correlated with benzenoid compounds and in particular with acetovanillone. Moreover, the authors were retrieving a correlation among skin-contact, the concentration of these compounds and the evolution of these descriptors. Other descriptors, like "coffee" and "dried figs" were also found to change in macerated wines.

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Table 2. Long skin contact effect on the level of several monoterpenes and norisoprenoids. Data are expressed in µg/L. SD: standard deviation

	Garganega								*Verdicchio*			
	White Wine		*Macerated Wine*		*White Wine*		*Macerated Wine*		*White Wine*		*Macerated Wine*	
	2008				*2009*				*2008*			
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Linalool	5.3	1.5	5.3	1.9	4.2	0.9	7.5	1.1	4.5	0.7	5.4	0.5
Ho-trienol	nd	nd	nd	nd	0.5	0.1	1.7	0.4	1.0	0.2	0.9	0.2
α-Terpineol	2.1	1.2	2.2	0.7	3.3	0.2	3.9	0.6	7.3	1.3	8.6	0.6
Citronellol	3.3	1.4	6.7	2.0	2.4	0.5	10.8	1.3	1.2	0.3	3.1	0.4
Nerol	nd	nd	2.7	0.7	nd	nd	2.5	2.2	nd	nd	nd	nd
Geraniol	3.1	0.2	4.7	0.1	0.7	0.1	5.7	1.6	nd	nd	2.2	0.5
Linalool oxide A	nd	nd	1.2	1.7	nd	nd	0.0	0.0	nd	nd	nd	nd
Linalool oxide B	nd	nd	0.8	1.2	nd	nd	0.0	0.0	nd	nd	nd	nd
Linalool oxide C	1.9	0.5	5.9	2.1	1.9	0.3	8.0	0.7	1.5	0.4	3.6	0.5
Linalool oxide C	nd	nd	nd	nd	nd	nd	0.0	0.0	nd	nd	1.0	0.2
Diendiol 1	9.1	0.9	7.4	1.7	18.9	2.6	31.2	2.5	7.2	1.1	7.0	0.2
Endiol	4.4	1.4	10.0	7.2	7.7	1.2	5.1	0.4	8.1	0.9	9.3	0.6
4-Terpineol	0.5	0.7	0.5	0.7	nd	nd	0.1	0.3	2.4	0.4	2.9	0.3
β-Damascenone	8.4	0.8	3.6	1.6	4.3	0.1	4.4	1.1	1.7	0.3	3.1	0.5

	Garganega								*Verdicchio*			
	White Wine		*Macerated Wine*		*White Wine*		*Macerated Wine*		*White Wine*		*Macerated Wine*	
	2008				*2009*				*2008*			
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
TDN	0.2	0.3	0.3	0.2x10^{-2}	1.2	0.5	0.2	0.2	0.7	0.1	0.3	0.3
Ethoxy-TDN	nd	nd	nd	nd	0.3	0.2x10^{-1}	nd	nd	nd	nd	nd	nd
Vitispiranes	nd	nd	nd	nd	1.7	0.3	nd	nd	nd	nd	nd	nd
Actinidoles	3.8	0.4	7.4	4.7	16.3	1.9	6.4	1.4	18.0	3.5	20.6	1.8
3-oxo-α-ionol	20.5	2.9	90.9	32.8	23.7	2.9	81.2	6.3	54.8	6.0	119.5	4.6

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Table 3. Long skin contact effect on the level of several volatile compounds (e.g. benzenoids, lactones, etc.). Data are expressed in µg/L. SD: standard deviation

	Garganega								*Verdicchio*			
	White Wine		*Macerated Wine*		*White Wine*		*Macerated Wine*		*White Wine*		*Macerated Wine*	
	2008				*2009*				*2008*			
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Methyl salicylate	1.5	0.2	6.7	3.6	6.4	2.3	33.9	14.6	86.5	5.7	161.6	35.5
Benzyl alcohol	52.8	5.8	479.8	197.3	90.4	15.3	982.6	228.8	399.1	86.2	863.6	205.8
2-Phenylethanol	41657.2	6803.8	54455.7	27491.5	27980.1	15364.4	44960.3	1850.7	24342.1	1026.7	23931.5	361.6
Benzaldehyde	5.4	2.0	8.9	5.6	4.6	2.7	16.4	1.1	17.7	3.6	296.2	103.2
Homovanillic alcohol	97.0	42.3	1097.2	308.4	119.7	25.4	1482.1	227.7	36.5	5.2	189.6	3.4
Vanillin	2.9	0.4	51.3	59.4	4.7	1.0	12.8	5.5	5.6	1.4	117.3	148.6
Phenol	1.4	0.4	4.3	1.4	1.5	0.4	4.0	0.1	3.2	1.0	5.0	0.5
Syringaldehyde	1.8	0.5	75.4	100.8	1.9	0.7	37.4	20.8	1.9	0.4	177.6	253.2
Acetovanillone	171.3	43.3	289.5	42.5	192.5	18.6	327.7	18.9	133.7	18.6	177.5	13.2
Propiovanillone	2.1	0.1	18.7	9.9	3.4	0.7	12.0	1.3	4.3	1.4	17.8	12.6
Butyrovanillone	7.9	8.7	53.3	65.3	4.6	0.9	19.7	2.2	47.1	4.6	271.4	35.8
Acetosyringone	2.1	0.2	7.8	3.0	2.3	0.6	7.4	0.7	13.1	1.2	35.2	11.5
Propiosyringone	2.9	2.2	7.7	4.3	1.2	0.2	4.4	0.8	0.9	0.2	6.9	6.1
Butyrosyringone	0.5	0.6	16.9	22.3	0.8	0.3	1.7	0.8	1.6	0.5	25.0	31.3

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	Garganega								*Verdicchio*			
	White Wine		*Macerated Wine*		*White Wine*		*Macerated Wine*		*White Wine*		*Macerated Wine*	
	2008				*2009*				*2008*			
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Tyrosol	1007.6	79.5	8744.2	672.3	804.4	135.6	3935.6	154.7	1522.6	40.7	11413.8	358.9
γ-Nonalactone	3.2	0.0	15.1	5.9	3.3	0.7	14.0	2.5	5.7	0.9	13.8	0.5
γ-Decalactone	0.6	0.8	0.5	0.6	nd	nd	0.0	0.0	nd	nd	1.4	0.2
γ-Butyrolactone	683.8	106.8	1263.3	370.4	818.1	103.6	2801.2	714.9	1487.8	153.3	2016.7	77.7
4-Carboethoxy butyrolactone	421.8	13.8	444.7	254.8	840.9	112.7	509.9	41.9	613.2	103.9	719.9	73.8

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Table 4. Long skin contact effect on the level of several fermentative sulfur compounds (data are expressed in µg/L). SD: standard deviation

	Garganega								*Verdicchio*			
	White Wine		*Macerated Wine*		*White Wine*		*Macerated Wine*		*White Wine*		*Macerated Wine*	
	2008				*2009*				*2008*			
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*	*Mean*	*SD*
Dimethyl sulfide	2.5	0.2	4.8	0.6	1.4	0.2	5.5	0.4	2.1	0.7	6.2	1.0
Diethyl sulfide	3.4	0.1	6.6	0.4	2.6	0.7	6.3	0.5	2.2	0.5	7.1	0.8
Dimethyl disulfide	2.2	0.3	3.9	0.5	2.1	0.7	3.4	0.9	1.8	0.7	4.4	1.2
Diethyl disulfide	1.5	0.4	2.0	0.2	1.9	0.2	2.4	0.2	1.3	0.1	2.5	0.7
S-methyl thioacetate	5.5	0.8	8.9	0.8	3.7	0.3	5.9	1.2	3.4	1.0	6.6	0.4
S-ethyl thioacetate	2.2	0.6	3.5	0.4	1.6	0.8	4.5	0.8	1.7	0.4	4.1	0.9
2-Methylthioethanol	10.2	1.2	27.2	2.2	7.8	1.2	30.1	8.2	5.6	1.6	25.4	5.2
3-Methylthiopropanol	1342.6	236.5	2536.5	264.2	1388.1	189.2	1809.4	210.9	973.3	179.2	1636.9	107.2
4-Methylthiobutanol	66.1	10.6	120.3	33.2	57.5	14.2	132.2	11.9	62.3	13.2	114.5	34.5

On the other hand, “floral” and “apple” descriptors were perceived significantly weaker in the macerated experiments. This evidence is in agreement with the common winemaking experience and also with the analytical data provided. In particular, fermentative esters significantly in macerated wines were significantly higher than in wines produced according to the traditional winemaking protocol.

Even though some of the sulfur compounds were sometime higher than their sensory threshold (e.g. MTP, DES), none of the panellist described the wines with descriptors that are usually associated with these molecules. Nonetheless, it cannot be excluded that these molecules are still playing an important role in defining wine aroma.

Georgian winemaking appeared to be a successful approach in producing white wines with peculiar characteristics and that attracted winemaker attention (data not shown).

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Chapter 9

Wine of Northwest China and Its Aroma Research Progress: A Review

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The winemaking industry of the Northwestern China is extremely important to the whole Chinese winemaking industry and development. From the ancient time when Qian Zhang missioned to the western region of China to the beginning of 21st century, the winemaking industry of China was continuously expanding. Especially, the growth rate has been speeded up rapidly since 1949 the year of the foundation of the People Republic China. This article is a review of the winemaking industry in the northwestern region of China which includes Shaanxi, Ningxia, Inner Mongolia, Gansu, and Xinjiang provinces. The effects of their geographic and climate characters on the quality of grapes and wines were summarized. Also, the distinctive sensory characteristics of the wines and local grown grape varieties of those regions were discussed in this review.

Keywords: China; wine; grape; sensory

Introduction

China has a long history of winemaking and wine culture. An ancient winemaking site as early as 9000 years ago, at the archeological research site of Jiahu, Wuyang County, Henan province, was discovered by the Chinese and American archeology scientists. During the reign of Emperor Hanwu, Qian

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Zhang was sent to the western regions of China as an envoy of Emperor Hanwu. When he was back, he brought the grape of *vitis vinifera* L., the most popular grape variety in the world. From then on, the grape vine and winemaking were developing with the attention of Emperor Hanwu. After the development of more than 2000 years from Emperor Hanwu period to the end of Qing dynasty, the winemaking industry of China had experienced the stages of starting, developing, and accomplishing. The winemaking industry and wine culture was always in maintaining, developing, and spreading whenever the history was in trouble or prosperity.

After the establishment of the People Republic China, especially in the last couple of decades, Chinese grape and winemaking industry had successfully developed. The development was attributed to the Chinese economic reform and collaboration and communication with the western developed countries. Between the late of 1950s and the early of 1960s, several hundred of table grape cultivars were introduced into China from Bulgaria, Hungary and the former Soviet Union. Since 1980s, some famous wine grapes in the world were introduced into China again. Meanwhile, great progress was made in the grape breeding technique in China. Thanks to the effort of all workers in the grape and winemaking, the special regions of Chinese winemaking have been formed. The regions are composed of Bohai, the old route of the Yellow River, Huaihe River basins, the dry climate and loess plateau area in Gansu and Xingjiang provinces, the south area of the Yangtze River, and the mountainous areas in the Southwestern China. In addition, Chinese wild grape wine regions have also been established. The regions are the Northeast China, Guangxi province and Hunan province, with the grape cultivars of *Vitis. amurensis*, *V. yeshanensis*, *V. lanata*. In recent years, the yield and consumption of the wines in China have had a rapid increase while the global wine consumption was continuously declining.Wines are becoming one of the most important alcoholic beverages in China.

The Wine of Shaanxi Province

Shaanxi province (Figure 1) is situated in the center of China and in the middle part of the Yellow River. It had been the center of the Chinese ancient commercial and culture center for thousands of years. Its east is separated from Shanxi province by the Yellow River and the west is jointed with Gansu and Ningxia provinces. It shares the north border with Inner Mongolia province. Its south area is linked with Sichuan province and Chongqing city. Henan and Hubei provinces are next to its southeast area. Shaanxi province likes a gate or passage linking both China's east and west areas which provides an unique geographical advantage.

Xi'an city of Shaanxi province became Chinese ancient capital 2100 years ago. The grapes (*Vitis vinifera*) were imported to Xi'an from Dayuan by Qian Zhang in Emperor Hanwu's reign. During the Tang dynasty, the grape planting and winemaking began to prosper. It was reported that the Emperors in the Tang dynasty, Tang Gao Zu (Yuan Li) and Tai zong (Shiming Li) loved grape-wine deeply, and today's Weibei HanYuan grape production area was Li's dynasty vineyard.

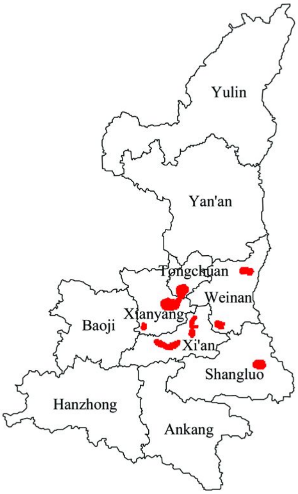

Figure 1. Shaanxi grape wine region.

Several winemaking factories with more than a hundred years history were located in Shaanxi province. They have also been rapidly developing to large scale winemaking enterprises in recent years. There are Danfeng winemaking factory, Yangling Tang dynasty winery, Hutai winery, Changyu (Jingyang) winery, Heyang winery, Yuchuan winery, and Tongchuan Kaiwei winery. There are many grape varieties in Shaanxi province. The traditional grape varieties are Longyan grape which was imported from France, Cabernet Sauvignon, Merlot, Cabernet Gernischet, Weibei No.1, etc. The main white grape varieties are Chardonnay, Riesling. In addition, some grape varieties were developed in China, they are Ecolly for white wine, "Meili" for rose winemaking, and Hutai No.8 for ice winemaking.

Shaanxi Danfeng Grape Wines

In 1911, during the Xuantong period of the Qing dynasty, Italian missionary Ann Seaman arrived in China and founded the Shaanxi Danfeng winery at the beautiful southeastern city of Qinling (Figure 1). Ann seaman and his apprentice took Longyan grape from the local ancient Danfeng Longju village as a raw material and adopted Italian winemaking techniques. They made the first bucket of grape wine which was ruby color, crystal transparency, and fully mellow savory. Shaanxi Danfeng winery has become one of the three oldest wineries in China. It has production capacity over 10,000 tons and storage capacity about 20,000 tons. There are different types of wine storage equipments, such as ork barrels, underground storage pools (6,000 m^3) and metal wine cans in

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Danfeng winery. Dozens of different brand grape wines: “Danfeng”, “Danjiang”, “Tianyun” and so on, were produced in this winery. The wines from Danfeng winery are becoming famous and popular in Shaanxi province and the northwest region of China (Table 1), and they bring huge economic benefits for Danfeng winery and make it to expand the production scale well.

Table 1. Main products and sensory characters

Main red wines: Cabernet Sauvignon, Cabernet Gernischet, and Grenache Noir	ruby red, clear, transparent, strong fruity and rich aroma, full-bodied, plump and ripe aroma
Dan Feng dry white wines: Ugni Blanc and Chenin blanc	grain stem yellow, clear, transparent, strong fruity flavor, and pure and fresh aroma
Dan Feng traditional red wines made from Longyan grape	palm, clear, transparent, fruity flavor with harmony, full bodied, comfortable sour and sweet aroma
Dan Feng Wu-wei wines made from Longyan and wild grape	strong aroma, ruby red, clear, transparent, fruity flavor, sweet and sour with longer aftertaste

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Weibei Hanyuan Grape Wines

Shaanxi Weibei Hanyuan (Figure 1) area is a valley region located in the Northern Shaanxi. The total area is 750,000 acres. The cultivated ground occupied 62.83% of the total area and is recognized as the perfect area for grape cultivation. The vineyard was planted in loess plateau (altitude 600~1300 m) with the slope of 6°. Different slopes and altitudes provide a mountain microclimate which is suitable for growing different varieties of wine grape. The accumulative temperature is 25.17~34.12 °C in summer, and it is higher than 30.0 °C in most of the region. The day-night temperature difference is 12~14 °C. The sunshine time is 1900 to 2533 h per year. The frost-free period is from 190 to 220 d. The annual precipitation is 550 ~ 730 mm. In general, it is a distinct and unique grape production region in China with a warm temperate and semi-arid, semi-humid continental monsoon climate. The soil is highly permeable and deep which is favorable for grape rooting, and the content of organic matter and mineral is adequate for grape growing and ripening.

Jingyang County is located in Guanzhong plain and the downstream area of Jing River. Its altitude is 550 to 700 m. It has the reputation “the core of cabbage” of the Guanzhong plain which means tender climate and fertile soil. Its climate is gently warm with continental monsoon. The average temperature is 13 °C per year. The average annual rainfall is 548.7 mm. The average annual sunshine time

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is about 2195.2 h. The frost-free period is an average 213 d per year. Since it has deep and warm soil and adequate light time and relatively great temperature difference of day and night, this county is one of the best areas for developing wine grapes. The towns, Baiwang, Kouzhen, Xinglong, Jianglu, and Longquan located in the northern mountain area of Jingyang County have the west-east length 35 km and the north-south width 5 km. The those places, there are 16,500 acres for planting high quality wine grapes. Right now, only 20% of the area is for planting wine grape.

The largest winery industry in China, Changyu Group, relies on the abundant grape resources of Jingyang County, have invested 1,600 million Yuan to build the Jingyang grapes winemaking company and introduced the first product wine on April 8, 2002. The main products are Bainian dry red wine, Cabernet Sauvignon dry red wine, QinJin dry red wine, Duoleyi sweet wine, and wide grape red wine, etc. (Table 2).

Table 2. Main products and sensory characters

Changyu dry red grape wine	ruby red, pleasant aroma, soft mouthfeel, smooth taste, strong structure feels, and lasting aftertaste
ChangYu wide grape red wine	ruby red, clear, transparent, fresh, strong fruity bouquet, and sweet aroma and moderate sour
ChangYu rose grape wine	shining color, clear, transparent, fresh and strong fruity bouquet aroma, full-bodied, and balanced harmonious
ChangYu Bainian dry red grape wine	ruby red color, clear, transparent, pleasing aroma, light oak aroma, soft mouth-feel and smooth.

Qinling's Hu County Hutai Ice Wines

Hu County at the north foot area of the Qinling Mountain is a very good place to cultivate Hutai grape for ice wine (Figure 1). The soil in the region is slightly acidic, gravel, and high in mineral content. Average annual temperature in the region is 13.4 °C, annual rainfall is 500~800 mm, sunshine time is 2000 h, temperature difference between day and night is 13~17 °C, and frost-free period 210 d. The accumulative temperature accumulation is up to 3,500 °C during grape growing from April to September. The temperature drops slowly from November to December. The air humidity is high and helpful to grape berry frosting. In the middle of December to the middle of January, the climate is affected by the Qinling Mountain's microclimate and cold current. The temperature before dawn can drop to -8 °C or more which only lasts 6~7 h for about 10 d.

In January 2008, Hutai No.8 grape was selected by Xi'an Grape Research Institute (Shaan Xi, China) from a French-American hybrid cultivar "Black Olympia"(Kyogei × Kyohõ). The grape is used for the ice wine in Hutai County. The volatile compounds profiles in the musts from the ripen grapes and the

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grapes frozen naturally on the vines were studied. The must samples were extracted using liquid-liquid extraction method. The extracts, concentrated by a rotary-evaporator and then pure nitrogen stream concentrator, were analyzed by gas chromatography-mass spectrometry (GC-MS). Total 68 volatile compounds were identified in both of the must samples. Aalcohols, ketones, esters and fatty acids were the main volatile compounds in Hutai No.8.

Hutai ice wine made from high quality Hutai grape with advanced and unique winemaking technology. It has golden color, transparent pure, elegant, full-bodied, honey aroma (commonly known as Guaizao aroma), tender and refresh taste, and unique wine structure.

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Guanzhong Plain and Shentang Winery

Shentang winery (Figure 1) is the only one winemaking which is technically supported by College of Enology of the Northwest A&F University and located in the Agricultural High-tech Industries Demonstration Zone−Yangling Demonstration Zone. The advanced science and technology of the college plays an important role in its wine research, production, and marketing and wine culture development. Meanwhile, it is also the base of comprehensive winemaking training and winemaking demonstration. It is a platform to improve wine scientific research and technology. The winery also has the missions to introduce the advanced technology and equipments and teach wine science and knowledge. It holds international winemaking conference every year. It is becoming a bridge between Chinese and foreign countries winemaking industries. This winery has trained enormous wine-tasters, wine-makers, and wine-technicians who are becoming the important personnel in the grape and winemaking industry in China. With the rigorous quality control "From the field to the table", a perfect internal quality control system was set up. It makes the grape wine from Shentang winery have great reputation in China. The Main products are listed in Table 3.

Ecolly Wine Grape

Ecolly wine grape was developed by the College of Enology of the Northwest A&F University, from Chardonnay, Riesling and Chenin Blanc as parents using the Eurasian recurrent selection method during 1982 to 1988. This grape variety was approved by the 22th testing of Shaanxi Crops Examination Committee on February 19, 1998. Li et al.(2005) (*1*) reported that Ecolly has strong resistance to mildew, black blain disease, and white powdery mildew. Meanwhile, it has good resistance to low temperature. This grape variety is suitable for making high quality white wine.

The volatile compounds of Ecolly dry white wine were extracted and identified by GC-MS. Alcohols and esters compounds were dominant volatile compounds and contributed the aroma of Ecolly dry white wine. Li et al. (*1*) reported that Ecolly white wine has a strong, enjoyable, special rose aroma, and elegant frangrance, tropical fruits flavors (melon and mango), nuts aroma, pure

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volatile, and balanced aroma. The quality of Ecolly white wine is better than Riesling or Semillon white wine.

Table 3. Main products and sensory characters

Li Hua "Shengtang" winery Chardonnay dry white wine (2007)	straw yellow, transparent, strong fruity flavor, linden flowers aroma, fry almond aroma, refreshing, downy, sweet, and lasting aroma
Li Hua "Shengtang" winery "Meili" rose wine (2010)	rose red color, transparent, elegant and rose flowers aroma, soft and tender taste, and longer aftertaste
Li Hua "Shengtang" Cabernet Sauvignon dry red wine (2008)	dark ruby red, with purple, strong aroma, blackcurrant flavor with spices, smoked, mushroom, turpentine, and full structure, longer aftertaste and good for aging wine
Li Hua Cabernet sauvignon-Merlot dry red wine (2001)	ruby red with yellow tint, elegance aroma, pepper, spices, smoked, mushroom, turpentine, smooth and fatty taste
Li Hua Grenache Noir-Melort dry red wine (1998)	dark brick red, fragrant and elegant, rich vanilla, mushrooms, cheese and meat, and sweet plum flavor, smooth taste, and longer aftertaste

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"Meili" Wine Grape

A new wine grape variety "Meili" was developed by the College of Enology of the Northwest A&F University from Merlot, Riesling and Muscat using the Eurasian recurrent selection method during 1982 to 1999. This grape variety was approved by the testing of Shaanxi Crops Examination Committee on August 11, 2010. Hua Li and his team reported that the character of "Meili" wine grape is stable and has strong resistance to diseases and high quality. It is a very great grape variety for rose wine.

Li et al. (2007) extracted the volatiles compounds of the grape wine by liquid-liquid extraction and determined the relative content of each important compound. The identified 31 volatiles compounds mainly included alcohol, esters and heterocyclic compounds (*2*). Also, there were several short-chain fatty acid esters which give fruity aroma, green apples aroma, banana flavor, brandy flavor. Especially, ethyl caprate provided brandy aroma, fruity sweet, and sweet grape aroma which are the main characteristics volatile of the wine. Heterocyclic compounds (thiophene compounds, pyrrole compounds and furan compounds were found in the dry red wine as well (*2*).

The wines made from "Meili" grape were composed of rose wine, sparkling wine, and red wine using different winemaking practices. Hua Wang and her team identified 57 aroma compounds from "Meili" wines by a SBSE-GC-MS method. Their volatile compounds were extracted using stir bar sorptive extraction (SBSE) method and then analyzed by gas chromatography-mass spectrometry (GC/MS).

Fifty seven flavor compounds were identified and quantified. Ethyl acetate, ethyl 2-methylpropanoate, ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, isoamyl acetate, octanoic acid, nonanal, isoamyl alcohol, 2-phenyl ethanol, linalool, β-damascenone, and β-ionone were identified as impact aroma compounds. For these impact odorants, “Meili” red wine had highest concentrations of the aroma compounds, linalool, β-damascenone, and β-ionone, while the sparkling wine had the lowest. “Meili” sparkling wine had the highest amounts of ethyl butanoate, ethyl hexanoate, and octanoic acid. However, “Meili” rose wine had the highest amounts of ethyl acetate, isopentyl acetate, ethyl octanoate, and 3-methylbutan-1-ol. In addition, higher amounts of 2-nonanone and δ-dodecalactone were observed in the sparkling wine. Although lower than the sensory threshold, these compounds may be the impact ordorants in the sparkling wine as well.

Sensory analysis showed that Meili rose wine had rose red, glittering transparency, elegant fruity flavor, rose flowers aroma, soft and smooth mouthfeel, and lasting aftertaste.

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Ningxia Province Wineries

Ningxia province (Figure 2) is located in the northwest area of China. About 1000 year ago, the grapes were planted in the region. Ancient poet Guan Xiu wrote a famous poem: “Red falls on the syzygitic leaves and fragrant flowers of licorice” which implied the grape was planted in Ningxia province. Yuan dynasty poet Mazhu Chang also wrote a famous poem named “Lingzhou” and stated “The fond wine grapes are living on the alfalfa fields”.

Ningxia province is far away from ocean and located in west of China. It has the typically continental climate. There is enough sunshine and temperature difference between day and night 10~15 °C. There is a saying goes, “wearing shirt early afternoon; dressing fur coat at night and eating watermelon round the fire”. The temperature difference is very helpful for the accumulation of sugar, pigment, phenolics and aroma substances of grapes. As the annual precipitation in the region is low, few diseases and pests are found in the grapes. The water from Yellow River is used from grape irrigation. However, the climate limits the latest maturity of the grapes. The frost-free period is short and about 160 days per year. The soil buried protection against cold needs in that area.

Modern wine industry of Ningxia province started at Yuquanying farm. In 1982, Yuquanying farm has 500 acres of grape planting field and produced the first batch of Yuquanying wine. It is one of the best wine grape producing are with effective accumulative temperature of 1534.9 °C (average $\geq$ 10 °C), effective accumulative temperature from July to september of 961.6 °C. The annual rainfall is about 193.4 mm per year. On April 11, 2003, the General Administration of Quality Supervision, Inspection and Quarantine of the People’s Republic of China (AQSIQ) approved the grape wine from the east area of Helan Mountain as the National Geographical Indication Protection Award.

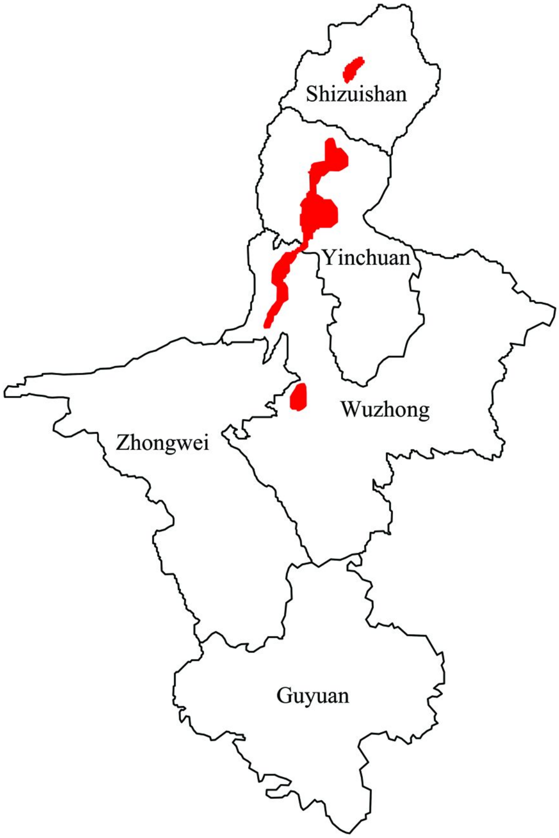

Figure 2. Ningxia grape wine region.

Up to now, Ningxia province has 18 wineries including Xixia King, Helan Mountain, Yuma, Jiabeilan, Hequan, Changyu, etc. The annual processing capability is 80,000 tons, And the planting field of wine grape is 32,900 acres. One third of them was newly built in 2009. It has formed the East Helan Mountain Grape Production Area. Qingtong City, Yongning County and Nongken Farm are the main grape production areas fellowed by Hongshibao County. The main red wine grape varieties are Cabernet Sauvignon, Cabernet Fran, Gernischet, Melort, Pinot noir, Syrah, Gamay, etc. The main dry white wine grape are Chardonnay, Riesling, Italian Riesling, Semillon, Pinot Blanc and so on. They produce dry red wine, dry white wine and sweet wine through natural brewing and traditional processing.

Chardonney dry white wine is made from its chardonnay grapes at low-temperature fermentation. It has crystal light straw yellow, strong fruit flavors, lime tree flower smell, Robinia flowers aroma, lemon aroma, grapefruit smell, and ripe pears and nectarine frangance. It also has almond smell, flavor elegant, fresh, wine strucure full and aftertaste lasting characteristics.

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Hua Li, Chun-long Yuan studied the sensory quality of the Chardonnay in Ningxia. It's color is straw yellow, green straw yellow, gold straw yellow, clear, transparent, and luster. It's aroma is fresh, pleasant, pure, strong, flowers aroma, lemon aroma, fruits flavors elegant, typical bitter almond smell and linden flowers flavors. It is also smooth in mouth, relax, harmonious, slow changing, harmonious, lasting; slight sweet, soft and smooth, little bitter, lasting aroma, aftertaste lasting, and balanced flavors.

Li et al. (2004) analyzed the aroma compounds of the Chardonnay in Ningxia by liquid-liquid extraction, 33 volatile compounds were separated and identified by GC-MS (*3*). The main compounds are alcohols, esters, and short chain fatty acids. Aromatic alcohols also played an important role in the wine due to their low aroma threshold. Those compounds contributed the special aroma with flowers flavors, rose flowers, violet, jasmine, spices pungent, mineral, anise, clove and fruits flavors.

Cabernet Sauvignon red wine in the region is dark red with vivid purplish red, mulberry, rich fruit, black currant, black cherry, red fruit, toasty oak and vanilla aroma and thick and mellow taste.

Zhao et al (*4*) compared Cabernet Sauvignon (2007) and Merlot wine (2007) from the Helan mountainous area of Ningxia and Shacheng of Hebei province. Fifty different volatile compounds were detected including phenolic acids, esters, alcohols, furans, aldehydes, and ketones. The authors also showed that Hebei Cabernet Sauvignon wine contained isobutyl acetate, 9-fullerenes sebacic acid ethyl ester, and thiophene ketone. However, the wine from Ningxia Helan mountainous area with varieties of wine did not contain thoese compounds. The Cabernet Sauvignon wine had higher level of ethyl acetate and lactic acid ethyl ester than Merlot wine (*4*).

Gernischet wine aged in oak barrels from Xixia king has inviting deep ruby red with baking oak and black berries aroma. It also has smoked, spices and vanilla aroma with special sensation and full-bodied taste.

Li et al. (*5*) studied the chemical constituents of the volatile comppounds of the Cabernet Gernischet by GC-MS. The main aroma components with higher relative content in the wine include 1-butanol3-methyl, butanedioic acid, propanoic acid, acetic acid, ethyl ester, etc. Hu et al. (*6*) also studied the Gernischet wine (2002). 49 wine aroma compounds were detected.

In addition to the Chardonnay, Cabernet sauvignon, Gernischet, the Ningxia region also produces high quality Riesling dry white wine, Pinot Noir red wine.

The Riesling dry white wine is light straw yellow, lively and lime, floral and fruity citrus aroma with a fresh and gooseberry balance.

Western Xiawang Yuquan manor ice white wine made from riesling grapes and Qiong Jade pulp at 8 °C low temperature fermentation. It gives a pleasant golden yellow with lemon sweet.

The Pinot Noir dry red wine has brilliant ruby red with fruit and oak strong harmonization and vegetables, minerals, tobacco and meat aroma, soft and smooth, and aroma lasting. The Mabing red wine is ruby red, rich fruit aromas and delicate mellow taste.

Melort dry red wine has ruby color with purple hue, spices, plum, mulberry, cherry, black chocolate smell and coffee smells with soft taste.

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Inner Mongolia Province Wineries

The region of Inner Mongolia province exists four huge deserts which occupy about 60% of the total area of the region. Inner Mongolia region is in arid and semiarid zone and has high temperature gap between day and night. Thus, it provides an unique advantage to wine grapes. Inner Mongolia has more than 200 years of viticultural history with 3 main grape production areas in three counties, Chifeng, Togto and Wuhai cities.

Wuhai city is located in the east of Ordos Plateau. Its west meets the grassland, the south of Alashan area, Yinchuan Plain. Its north is near Hetao Bend. The length of from north to south is 69 km with width 42 km. The total area is about 2350 km^2. Wuhai city has typical north temperate continental climate and semi-arid, half desert climate, sufficient sunlight, annual sunshine hours for 3047~3227 h, average temperature of 9.6 °C, and low temperature-average frost-free period 156~165 d. The climate characteristics is high temperature in hot summer, short autumn season, long winter season and high night-day temperature difference. It lacks of rain and has the average rainfall 159.8 mm which is very suitable for grape growing.

Nearly 100,000 acres of arable land in Wuhai are available for developing more than 650000 Chinese acres of the sand loam land with gravel soil in 0.4~1 m. The soil pH is 6.8~8.0. It has good quality of water for irrigation. At present, the Wuhai grape planting area is 3, 294 acres and mainly for the varieties of Cabernet Sauvignon, Cabernet Gernischet, Baiyu, Aaron thickener, riesling, etc.

Hansen wine industry Co., LTD is in wuhai city and was founded in March of 2001. It has annuel wine production capacity 20000 tons and grape planting area 1, 650 acres. It consists of a collection of seedlings, organic grape planting, organic grape processing, and import and export marketing and is a greatly profitable wine enterprise. In 2007, the company's grapes and wines were approved as organic food and green food with grade AA certification. In the same year, it was named top 100 private enterprises in China, It is the first enterprise of Inner Mongolia province to obtain the Famous Trademark Award. In 2008, it is recongizned as the Model of National Farming and Meat Industry.

Hansen chardonney dry white wine is grain smell, yellow pole hazel sweet, flowers, lime tree with minor almond sweet full-bodied fragrance, and pleasant feeling.

Hansen cabernet sauvignon is red wine with deep ruby red, blackberry aroma, and a little berries taste. It also has spices, mushrooms, smoked aroma with distinct smell of tannin structure and longer lasting taste.

Hansen's snow and ice grape wine (sweet type) is golden yellow, aromatic flavor, mellow taste with icy taste.

Gansu Province Wineries

Gansu province (Figure 3) is located at the western of China and in the upper area of the Yellow River and Loess Plateau. It is next to Inner Mongolia Plateau and Tibetan Plateau areas. The region is long and narrow. The distance between its east and west is 1655 km. In China, Gansu province is one of the earliest grape

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cultivation areas. As far as 2400 years ago, grapevines were planted in Liangzhou (Wuwei present area). During the the reign of Emperor Hangwu, Qian Zhang was sent on a diplomatic mission to Xiyu (the western regions). He brought back grape seeds and introduced the winemaking technology from the western regions. Then the grape cultivation and winemaking began to appear in Liangzhou. The philosophers, poets and writers at that time were inspired by the Liangzhou grapes and delicious wine and wrote many amazing poems in the history. For example, the poem of "The song of Liangzhou" was written by Wang Han, Tang dynasty poet about the soldiers, battle field, and wines.

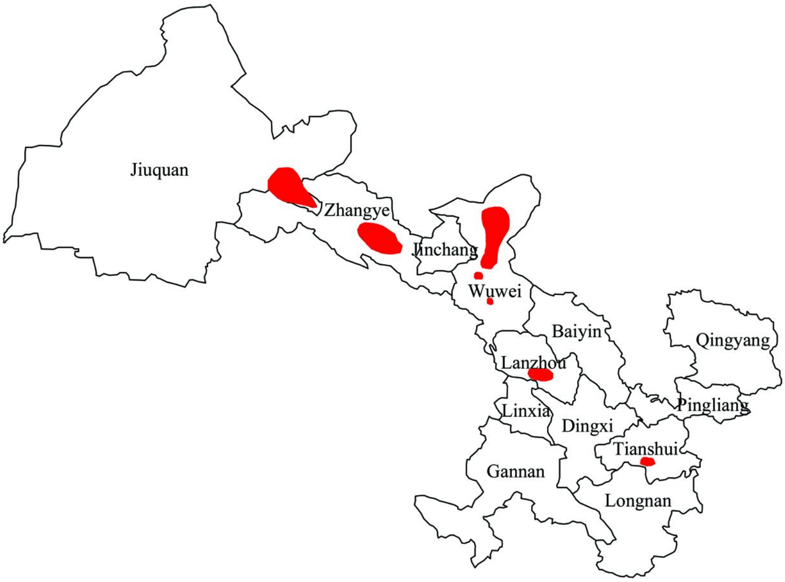

Figure 3. Gansu grape wine region.

The wine grape planting area and wineries of Gansu province are mainly located in Hexi corridor area. The Hexi corridor is also called Gansu corridor where is from the north of Qianlian Mountain to the south area of Beishan Mountain and the northwest area from the downstream of Shule River to the southeast area of Wuqiao Mountain. This region is the best district of grape planting area and has formed tree wine grape production areas including Wuwei, Zhangye and Jianyu Guan (Jiuquan). Tianshui City, and Lanzhou the capital of Gansu province.

In 1983, Mogao Co., LTD in the Ecological Agriculture Demonstration Area (former an antelope fruit farm) developed a planting area of 350 acres which is one of the first high-grade winery and was built by the Light Industry Department of China in 1980s. After 1997, the grape planting and wine industry has stepped in a quickly developing period and generated several distinguish features and promising future wineries, Zhixuan, Qilian, Guofeng Huangtai, etc. Up to 2008,

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the wine grape planting area of Gansu was about 20,000 acres, accounting for 17.5% of the total of China. The wine grape was about 120,000 tons, accounting for above 18.0% of the total China. Its wine production was about 80,000 tons, accounting for 3.45% of the total of China.

Gansu has a typically continental climate. Sunshine time is long and average of 2724.8 h in Wuwei. The effective accumulative temperature is 2800~3200 °C, and difference of day-night temperature is above 10 °C, especially from June to September about 14 °C. The frost-free period per year is from 48 to 228 d. The temperature in winter is low. The vines need to be soil buried in order to protect against freeze injury. Gansu is dry and lacks of water. The annual rainfall gradually increases from 30 to 860 mm from the northwest to southeast area. The average annual rainfall has been about 280.6 mm in Gansu province for many years. The annual rainfall of Wuwei is about 191 mm. The climate features of four seasons in Gansu is longer cold time in winter, temperature increasing sharply in spring; high temperature and rainfall in summer and temperature quickly decreasing in the early of fall.

Hexi corridor area in China is the best wine grape planting region. There is sandy and soft soil which is beneficial to root growth. The mineral content is high and suitable for the formation of grape flavor. The scarce rainfall and huge temperature difference of day and night are helpful for the sugar accumulation. The dry air reduces plant diseases and insects. The cold and cool climate is helpful to flavor substances accumulation during the grape ripening period.

Table 4. Main products and sensory characters

Mogao Riesling	light yellow with green hue, peach and citrus aroma, honey sweet smell, soft and fresh mouthfeel, and elegant after taste
Mogao Chardonnay	light straw yellow with green hue, clear, transparent, green apple and melon smell, citrus aroma, moderate acidity, soft, round and elegant taste
Mogao Pinot noir	light ruby red, clarify and luster, the strong tropical fruit flavors and roasted oak to coordinate, feeling harmonious in mouth, appropriate sour and astringent with light clear and sweet, licorice and cooked beets head flavor, soft and fresh taste, and aftertaste lasting

There are many wine grape varieties planted in Gansu. After the 20-years development, 12 grape varieties which are suitable in Gansu were picked up from 126 varieties. In general, Zhangye, Jiuquan and Jiayuguan are suitable for Merlot, Cabernet Sauvignon, Chardonnay, Limberger and Semillon. Wuwei is the best region of China for Pinot Noir. Quality wines from Merlot, Chardonnay and Limberger can also be produced in this area, but not Cabernet Sauvignon (Table 4).

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The middle area of Hexi corridor, Gansu province is under the Qilian Mountain and the northwest edge of Zhangye basin. The Qilian grape planting area is surrounded by Qilian and other mountains. The sunshine time is up to 3088.2 h with average temperature 7.6 °C per year and the highest temperature 38.7 °C and the lowest temperature is -31 °C. The average temperature difference is 14.9 °C per day. The accumulation temperature is 3039 °C per year which is equal or above 10 °C per day. The rainfall is between 66.4 mm and 104.4 mm. The underground water resource is rich. The mineral water from the Qilian Mountain irrigates grape. There are few pests and no industrial pollution. It is perfect to plant some famous grape varieties, Cabernet Gernischet, Merlot, Semillon Blanc, and Italian Riesling (Table 5).

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Table 5. Main products and sensory characters

Qilian Italian Riesling dry white wine	straw yellow, pure, fresh lemon, rose flowers and pagoda flowers aroma with smooth and lasting taste
Qilian Legend ice white wine made from Italian Riesling grape	light gold yellow, strong fruity flavor, elegant, and harmonious with fruit, flower honey and apple smell, and soft and round wine structure
Qianlian red ice wine made from Merlot grape	ruby red, light and pleasing color, sweet and mellow smell and deep and lasting after taste.
Qilian Cabernet Gernischet dry red wine	brick red, typical flavor of Cabernet Gernischet, strong fruit flavor, elegant and balanced oak flavor and mellow mouthfeeling, appropriate tannins, strong structure feeling, plump and taste lasting.

Zhu et al. (*7*) studied the aroma compounds in Italian Riesling dry white wine. Forty six aroma compounds were detected from the wine.

Gansu Zhangye Guofeng Wine Limited Liability Company was established in 2000 and has a production capacity of 10000 tons per year. There is a high quality grape production area over 1,650 acres, which is located in Banqiao Town, Linzhe County of Zhangye City and Pingyuanbao Town of Ganzhou District, Zhangye City. There is long sunshine time in this area. The temperature difference between day and night is significant with long free-frost time. The soil is sandy and soft soil with good ventilation, moderate pH, and few diseases and pests. It is one of the best ecological areas for grape growing. The main grape varieties are Merlot, Cabernet Franc, Chardonnay, Pinot noir, Syrah, Italian Riesling, Riesling and Cabernet Sauvignon, etc (Table 6).

Gansu Zixuan winery is located in Juayu Guan, the north area of the Qianlian Mountain. The climate is dry, enough sunshine time, and huge temperature difference between day and night with few diseases and pests. It has typical desert soil which is very suitable for wine grape. The advanced grape growing technology in this world like Israel drip irrigation, fertilizer technology and all-around stereo scientific cultivation were applied in Zixuan Company. Those technologies not only solved problems of insufficient water but also provided enough nutrients for the grapes as well.

Table 6. Main products and sensory characters

Guofeng five-star Cabernet Sauvignon dry red wine	dark ruby red, strong fragrant, strong fruit flavor, full-bodied and pleasant aroma, elegant and mellow, char, and elegant, quiet and pleasant smell with balanced and tender mouth-feeling, nice structure and aftertaste lasting
Guofeng Syrah wine	dark ruby red, mellow appearance, strong flavor of black-currant and mushroom. Its aroma is complete, comfortable, mellow and fresh with sugar plum and cherry flavor and long aftertaste.

Today there is 2,500 acres vineyard in Zixuan. Wine production scale is planned to 50,000 tons. The first-stage project scale of the construction is 10,000 tons per year. It owns the biggest underground oak wine cellar in China. Their great commitments and technology will provide extremely high quality grape wine from the central desert. This winery can make dry wines and ice wine using 20 grape varieties (Table 7).

Table 7. Main products and sensory characters

Zixuan dry red wine	red with purple hue fresh appearance and wild flower Fen violet flowers smell
Zixuan Merlot dry white wine	dark ruby red with purple, typical strong fruit flavor, spices, smoked , mushroom, turpentine smell, smooth and soft wine body and lasting taste.
Zixuan oak barrels Cabernet Sauvignon dry red wine made from Cabernet Sauvignon wine grape	dark ruby red, strong fruits flavors, with small black berries such as blackberry flavor, cocoa and dry plum flavor and other dry fruit flavors, mixed typical and elegant, full body, balance and mellow structure, fragrant and exquisite, and lasting taste

Song et al. (*8*) studied the main aromatic compounds of Cabemet Sauvignon wines from Hexi Corridor original producing area by headspace solid·phase micro-extraction and gas chromatography-mass spectrometry. The total 174 aromatic compounds were identified, including 53 esters, 41 alcohols, 18 organic acids, 15 terpenes, 12 hydrocarbons, 8 ketones, 14 aldehydes, and 13 others.

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Wine from Xinjiang Area

Shinjang Uyghur Aptonom Rayoni is usually called Xinjiang. It lies in the northwest of China, east longitude 73°20′~96°25′, north latitude 34°15′~49°10′, and area 166 km^2, accounting for 1/6 of the total area of China (Figure 4). Its west, north and northeast area is next to kyrgyzstan, kazakhstan, and Russia, respectively. Its southwest area is next to Afghanistan, Pakistan and India. The east is linked to Gansu province, and Hexi corridor. Its southeast area is adjacent to Qinghai province. Its south area is divided with Tibet autonomous region by Kunlun mountains. In history, it was the most important region of “Silk Road”, which linked up the eastern and western countries. Now it becomes the important place of the second “Euro-Asian continental bridge”.

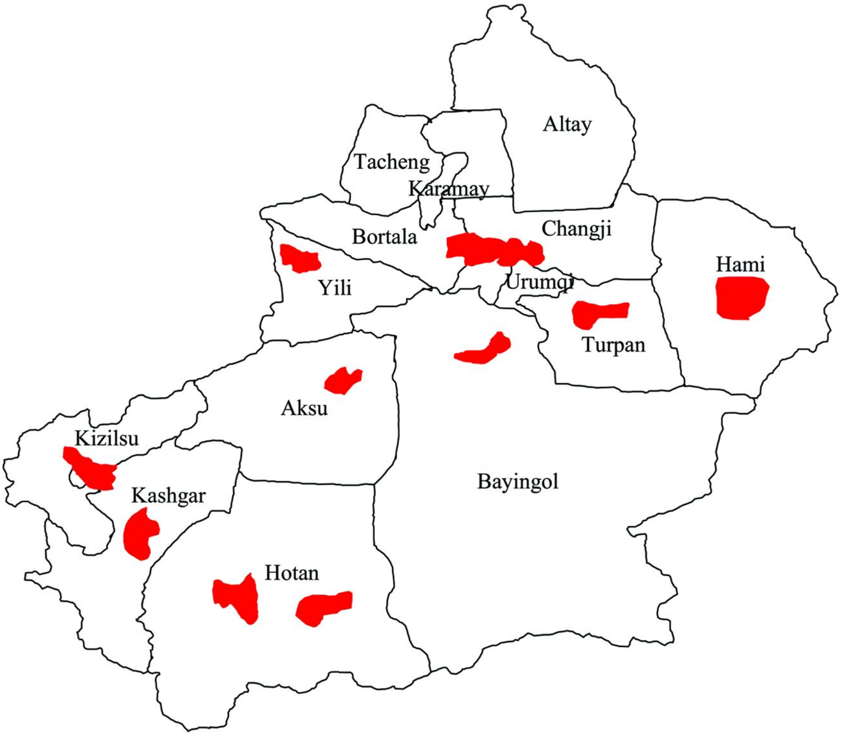

Figure 4. Xinjiang grape wine region.

In BC 138, Han dynasty ambassador Qian Zhang imported the grapes and firstly transported them to Xinjiang. Then they were through Hexi corridor of Gansu province to arrive Xi’an city of shaanxi province and other areas. The history of Xinjiang winemaking is over 2,400 years. With China’s modernization and implementation of the western wine technology, Xinjiang is one of China’s

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top 10 excellent wine grape production areas and has gradually formed their own wine characteristics and produced high quality grape wines.

Xinjiang has more than 40 wineries, most of them are small wineries which just only have 500 tons production capacity per year. Since the beginning of this century, the scale of production has increased dramatically and become the important wine production area in China. The Citic Guoan group, Loulan and Xiangdu wineries are well known.

Xinjiang province is far from the oceans. It is devided into two parts by Tianshan Mountain, with the north and south areas of Tianshan Mountain called north Xinjiang and south Xinjiang, respectively. The Tarim basin is located among the Kunlun Mountains with an area of about 53 square kilometers. It is the largest basin in China. The Taklamakan desert is located in the central part of the basin with an area about 33 square kilometers.

The climate of Xinjiang is typical continental climate due to the Tianshan Mountain can stop cold air moving into south Xinjiang. The Tianshan Mountain becomes the border of two different temperate zones. The southern Xinjiang is the warm temperate zone with average temperature of 10~13 °C while average temperature in north Xinjiang plain is below 10 °C. The extreme highest temperature was up to 48.9 °C in Turpan, while the lowest temperature was below to -51.5 °C in Keketuohai Fuyun County. The accumulative temperature of the year is more than 4,000 °C, which is more than 10 °C per day in the south Xinjiang plain and less than 3500 °C in the north of Xinjiang plain. In the south Xinjiang plain, frost-free period is 200~ 220d while most of the north Xinjiang plain is less than 150 d. The sunshine duration distribution from north to south is slightly reduced to 3001 h and increased to 2828 h from west to east. Rainfall in the north Xinjiang is less than the south Xinjiang. The annual rainfall is only 145 mm in Xinjiang which is accounting for 23% in China average rainfall per year (630 mm). Xinjiang's annual rainfall is the least one in the same latitude. Annual rainfall in the south side of the Tianshan Mountain is 20~400 mm, the north side of the Kunlun Mountain is 200~300 mm.

The climate in Xinjiang, such as are long sunshine time, huge accumulative temperature and the difference between day and night, long frost-free period, as well as soil is suitable for producing quality wine grape.

The north of Tianshan Mountain region is main flood alluvial plain which is gradually slant from the south to north. It is flat and open. The soil is alluvial deposit containing small size of gravel, sand, and soil particle. The soil is loose and permeable. It is very helpful for grape root growing. Soil is brown and grey desert which are rich in organic content (0.2%~0.8%), calcium and some other important elemensts such as total nitrogen (1.3% mg/g), phosphorus (5 mg/g), potassium (348 mg/g), boron (4.3 mg/g), iron (3.34 mg/g), molybdenum (0.5 mg/g).

The main Xinjiang grape cultivation areas are Turpan, Hetian, Hamilton, Changji, Yili, etc, with a total area 110000 hactare (0.27 million acres). The main red wine grape varieties are Cabernet Sauvignon, Cabernet Franc, Gernischet, Melort, Gamay, Syrah, Limberger, Le Pinot, and Saperavi. The main white wine grape varieties are Chardonnay, Riesling, Italia Riesling, Pinot Blanc, Chenin blanc, Costalupo Controguerra, and other 73 varieties.

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Yili Xinjiang Winery

Yili winery (Figure 4) is located in the Yili River valley. This region is in the west of the Tianshan mountain valleys and linked with Kazakhstan (longitude 81°26′~81°37′, latitude 43°49′~43°53′). The area includes Yili city, Huocheng County, the Xinjiang Production and Construction Corps and other corps. It is well known as "The Greenhouse of the Central Asia" and "Saiwaijiangnan". It has beautiful scene, magnificent landform, grassland, rivers, ancient smoke signaling towers, boundless forest, and abundant animal and plant sources. It has great biodiversity of the natural rare gene pool in the Asia inland Accumulative temperature is 3170 °C~4100 °C, sunshine time up to 2820 h It is an important grape production areas. Main red grape varieties are Cabernet Sauvignon, Canepabn, Cabernet Gernischet, Carignane; and the white grape varieties are Riesling, Chardonnay, Italia Riesling, Rkatsiteli, etc.

Yili winery is the first wine manufacturers in the region. After the forty years of development, it has introduced the high quality of wines with different varieties, Riesling, Italia Riesling, Carignane, Canepabn, Cabernet Sauvignon, Cabernet Gernischet, Cabernet Franc, Melort and Limberger and other (Table 8). The grapes production area is abount 494 acres. The factory covers an area of 40,000 square meters and a building area of 4,948 square meters. The annual wine production capacity is 3000 tons with 20 million Yuan assets.

Table 8. Main products and sensory characters

Dry white wine	fully clear connect and golden burnish with fresh fruity flavor, bouquet, pure and fresh, crisp pure and delicate, and full-bodied taste with a long aftertaste.
ice white wine	light golden brown, clear, transparent, fruity aroma, and pure, round and full bodied, lingering fragrance and aftertaste lasting.
ice red wine	ruby red, clear, transparent, strong fruity aroma with good balance.
dry red wine	deep ruby red, transparent, fruity and full bodied, elegant great satisfying and a lingering finish

Xinjiang Citic GuoAn Group Winery

Citic Guoan Group Winery (Figure 4) is located in Shihezi where is in the north of Tianshan Mountain and Junggar basin and the ancient southern desert Ku Er Ban. The total area is 1320 km^2, which 730 km^2 area can be planted. The varieties of ChangJi, Hiutubu, Manasi, and Shihezi grapes are planted between its region highway and 312 national highway.

In the Shihezi flood alluvial zone, soil can be divided into gravel soil and coarse silt loam soil. It has four seasons annual mean temperature 6.0~6.6 °C,

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frost-free period between 160~170 d, the most average temperature at Leng Yue 16.1 °C, annual precipitation 110~200 mm, annual sunshine percentage 63%, and deep and good permeability soil. The harvested berries have good quality, mature color, high sugar, moderate acidity, and light acerbity. The drought Manasi plain area has grape growing in cold climate which is accumulative temperature of 3200 ~ 3800 °C, an average temperature 21 ~ 22 °C in June to August, huge temperature difference between day and night, and the annual rainfall 200~300 mm. The general mid-late maturity varieties can be fully ripened at cooler climate during the later growth stage. It is good to the production of sugar and produce quality red, acid, dry white wine and champagne wines and some high quality Pinot Noir, Riesling, and Chardonnay.

Citic Guoan Group Winery consists of grape cultivation, processing, marketing, research and development. It is one of the major wine enterprises relying on the natural Xinjiang unique ecological resource and has developed to be the center of the grape special industry. The company has production scale 115000 tons and wine storage capacity of 150000 tons, and wine filling capacity of 80000 tons. Currently, it is the largest wine production enterprise in Asia.

Citic Guoan Group Winery in Xinjiang with 150,000 acres grape production area in the north of Tianshan Mountain and Yili River valley which is located in Bogurda Biosphere Reserve Area. The Tianshan Mountain Bogurda Biosphere Area is zero pollution ecological environment. The biosphere area in the north side of the Tianshan Mountain are mainly composed of three small ecological gardens: Tianchi Portuguese garden, Tianshan Mountain Tianchi Portuguese Garden, suitable to make fine wine grapes.

The products of the Citic Guoan Group Winery are controlled through the high standards of raw grape material and brewing, winemaker, storage technolgoy and other products quality standards. They are divided into luxurious level, collecting, special making level, 4 well making levels to provide consumers with safe, healthy and high quality grape wines (Table 9).

Table 9. Main products and sensory characters

Chardonnay dry white wine (collection level)	elegant light yellow, with grain stem pagoda, peaches, melons and pure and fresh flower fruit aroma, delicate oak, exquisite and elegant flavor, and liquor quality to the palate with a full coordination
Dry Riesling white wine level (Tian Mountain Tianchi Portuguese Park)	shallow grain, the dominant pole yellow green apple, orange pure and fresh fruity flavor, moisture and refreshing and distinct characteristics with cool and lasting taste.
Niya Cabernet Sauvignon dry red wine (luxurious collecting level)	pleasant ruby red color, typical plump with ripe fruit blackcurrant aroma, aged pulp and spicy oak fusion coordination, and palate and full-bodied with a long aftertaste.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

Chen et al. (*9*) analyzed the aroma components of the Chardonnay dry white wine by GC-MS and identified 49 volatile compounds. Xu et al. (*10*) studied the aromatic compounds in Cabernet Sauvignon wines from the Northern Foot of Tianshan Mountains in Xinjiang. The main compounds are isopypentyl acetate, monoethyl butanedioate, ethyl octanoate, ethyl butanoate, ethyl hexanoate, diethyl butanedioate, 3-hydroxy, ethyl butanoate, ethyl decanoate, ethyl, 2-hydroxy, propanoate, 1-hexanol, 2-hexen-1-ol, 3-methyl-1-butanol, phenylethyl alcohol, 2-methyl-1-propanol, etc.

Hua Li and Chunlong Yuan studied the sensory quality of Cabernet Sauvignon wine in the Xinjiang region (Urumqi) and concluded that 1) appearance: deep ruby red, deep red, dark red, dark purple, purple, clear, transparent, have wine column, luster and bright; 2) aroma: pure, full-bodied, elegant and pleasant sweet feeling, show slightly mushroom flavor, blackberry fruit, light oak flavors, pure and fresh fruit flavors, liquorices, and dark tea performer son sweet, sootiness flavor, little berries taste, smell, taste, fat cooking born green flavor, green stalks flavor, cedar smell, the pine is sweet, pepper taste, apricot flavor, strawberry; 3) palate entrance: fruity, plump, comfortable, change slow, the slower sour, tail taste slight bitter, coordinated, and tannin structure feels strong, balance, mellow, long, long lasting, mouth taste, smell, the coordination of the elegant, have skeleton, soft, a lingering finish, and aftertaste lasting.

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Xinjiang Loulan Winery

Xinjiang Loulan Wine Co., LTD. (Figure 4) is located in Turpan. This region is located in the east of the Tianshan Mountain. It has gravel soil and sandy loam which give good water permeability and salt level. It is a dry area famous for table grapes and seedless grapes. The region includes Turpan City, West County, Gongliu River. It is in a warm temperate zones with activities accumulative temperature of 4500~5000 °C, average temperatures as high as 28~34 °C, high night and day temperature difference. In the grape wine region, there are plenty of sunshine, while annual precipitation, from 20 to 50 mm, is low. The water of melting snow from Kan Er Jing and Tianshan Mountains was used from irrigation. The grapes of Cabernet sauvignon, Merlot, Cabernet Franc, Grenache, Syrah, etc. were planted. Due to the higher temperature, the sugar content of grape is about 25%~28% with low acidity. It is suitable for high-grade sweet wines with the western regions characteristics or for mix-varieties grape wines.

Xinjiang Loulan wine Co., LTD. is one of the leading enterprises in the Chinese wine industry. Its predecessor is Shan-Shan County Winery founded in 1976. It was invested by the British High Mountain Company in 1996. The winery is located in the east of the Flame Mountain in Turpan Basin. The vineyard planting area is more than 494 acres and high quality grape planting area is more than 165 acres. The Loulan winery selected European varieties wine grape as raw materials, planting in the Gobi desert, irrigating with no pollution water from Kan Er well under the Tianshan Mountain, applying the traditional processing technology to produce fine grape wine.

Loulan selected Chenin blanc dry white wine is shallow light yellow with green, clear, transparent, luster, pleasant pagoda and sweet, sweet honey by pure fruity, fresh, elegant, relaxed, and full-bodied.

Loulan selected Cabernet Sauvignon red wine is deep and bright ruby red, luster with red apple, cherry, pleasant fruity, oak and bouquet coordination with palate and lingering finish.

Hua Li and Chunlong Yuan described the Xinjiang region (Shan Shan) Cabernet Sauvignon wine 1) appearance: deep ruby red, purple, red, precious stones ruby red with purple adjusted, mulberry, micro purple, transparent, luster, has hang cup phenomenon, wine column; 2) aroma: pure, full-bodied, harmonious, fresh, elegant, quietly elegant, comfortable, with some pepper taste, the fresh flowers, grass green flavor, taste, born a blackberry aroma, fresh grass flavor, sweet, flavor like mushrooms, sootiness flavor, and dark tea performer fruit, chocolate flavor, small berries, with other dried fruit and earthy taste, cinnamon, ginger taste, smell cedar turpentine flavor, toast, licorice taste sweet, apricot taste; 3) taste: soft and smooth taste, alcohol, round and thick, sweet aftertaste, a bit of a rotund, tail taste bitter, liquorices, outstanding after taste bitter, rich, good sweet tannins structure feels, coordination, and good structure feels.

Xinjiang China-France Co-Investment Xiangdu Winery

Xinjiang China-France Co-investment Xiangdu winery (Figure 4) is located in Yanqi region which is in the South of the Tianshan Mountain (longitude 86°17′08″~86°21′56″, latitude 42°06′01″~42°03′03″). The total area of the region is 78 km^2 with 13 km length from east to west and 6 km width from north to south. It is 35 km from the city of Ku Er Le (43 km, capital of Bazhou). The National Highway 218 is through the area and divides it into the east and west parts. The area includes Ku Er Le City, Yanqi County, and Hoxud County. This region has the typical desert climate with scarce rainfall and long sunshine time, for example, annual average temperature is 8.9 °C, and effective accumulative temperature is 3188.5 °C~3449.5 °C, average sunshine time is 3128.9 h, annual rainfall 64.7 mm, annual evaporation 1194.7 mm, frost-free period more than 180 d, and huge temperature difference between day and night.

Due to it is the flash flood alluvial margin zone with an average elevation of 1100 m, the soil texture is sand and gravel with good permeability and pH of 7.5~8. The soil is coarse to fine and gradually increasing organic matter from its surface. However, the rainfall is scarce. The Kai Dou He Tianshan Mountains melting snow and groundwater resource is rich and adequate for grape growing in the area.

Xinjiang China-France Co-investment Xiangdu Winery was established in April 30, 2002 with the registered capital 20 million Yuan. The shareholders of the company now are Xinjiang Basin Assets Management Co., LTD, Xinjiang Development of Agriculture High-Tech Yanqi Instrument Co., LTD, and Hong Kong Blue Panda Development Co., LTD (Table 10).

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Table 10. Main products and sensory characters

Xiangdu dry white wine	straw yellow, strong fruity flavor, fresh and relax taste
Xiangdu dry red wine	red ruby red wine, with exquisite, raspberry and strawberry aroma, full-bodied harmonious and longer aftertaste
Xiangdu Cabernet dry red wine	pomegranate red, the silky, with gooseberry, blackberry smell and spices aroma, soft tannin and fine and smooth, stable, and longer aftertaste

Near the place there is another winery called Xinjiang Ruifeng winery. This winery is located in Xinjiang Quhui Hoxud County and was established in November 2000. Natural underground water reservoirs is sufficient in the area. The soil is gravel brown desert soil with sand. The pH value of topsoil is between 7.9 and 8.3. It has low organic content, poor nitrogen, phosphorus and potassium level. The annual precipitation in the area is 74.4 mm,while annual evaporation is 1194.7 mm. As the climate controlled by the waters in basin, the temperature change is not signficant. It has average temperature of 8.2 °C, the highest temperature 38 °C, the lowest temperature -35 °C, activity accumulative temperature 2978.5~3239.5 °C, frost-free period 185 d, annual sunshine hours 4440 h. The sunshine, soil, and groundwater give the advantages in growing high quality of grapes to the Ruifeng winery.

The Ruifeng Winery has followed the chateau wine production concept-high quality, small amount products, using local special geographic location and climate, using half-underground fermentation process and less mechanization, avoiding wine composition physics and chemical change, to ensure that the quality of the wine nature. Since December 2007, all the products of the Ruifeng Winery have been certified as “Organic Products” (Table 11).

Table 11. Main products and sensory characters

Ruifeng Winery Riesling dry white wine	Straw yellow, Glittering and translucent, bright beautiful, elegant aroma with a relaxed and harmonious taste
Ruifeng Winery Cabernet Sauvignon dry red wine (oak barrels caches)	delight ruby red color, aroma fragrant, plum rotund, with a long aftertaste.

Summary

China is becoming the main consumption country of the world wine. In recent years, grape production and winemaking industry have made signficant increase. In 2008, the grape cultivation area reached 449958 hectares, the fifth in the world. In 2009, the grape cultivation area increased to 458700 hectares. In 2008, the grape yield was 7151500 tons, the second in the world with the wine grape cultivation area of nearly 62700 hectares. According to the data released by the National Bureau of statistics, China's wine production reached 69.83 million liters in 2008. In 2009, the production increased to 96 million liters which was the world's seventh great wine producer. In 2010 China's wine production reached 108.88 million liters, ranked seventh in the world. The sales revenue was 28.5 billion Yuan with a profit of 3.3 billion Yuan in the period of January to November 2010. The wine industry has become China's important promising industry. In the recent 10 years, the Chinese winemaking industry has maintained high growth rate. The increase rates of the wine production by the previous year were 37.05% in 2007, 23.4% in 2008, 27.63% in 2009, and 12.38% in 2010.

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Chapter 10

Wine Oxidation: Recent Revelations, Observations, and Predictions

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While wine oxidation is one of the oldest problems in winemaking, it still has much current interest in the research and winemaking communities. Recent studies have demonstrated basic new information about the specific reactions and necessary catalysts in wine. Recent developments have shown that catalytic metals are essential, that the ethanol free radical is abundant in oxidized wine, that cinnamates are important antioxidants and that oxidation products are key to color development in red wine, among many new reports. Current winemaking includes deliberate use of oxygen to manage wine flavors and emerging research is likely to provide winemakers with new tools for managing the effects of wine oxidation.

Introduction

The microbially assisted oxidation of wine to vinegar was the limiting factor in wine preservation and use for the first 10,000 years of winemaking. Pasteur revealed the microbial agent in 1864 in his "*Memoire sur la fermentation acétique*" changing the fundamental knowledge of this problem. However, today's technology can almost totally exclude oxygen from wine production, and winemakers now have to determine an optimal amount of oxygen or wine oxidation to achieve the style they desire. The aging of wine was and still is also limited by its chemical oxidation, also described by Pasteur (*1*) where he showed that in an ampule, the wine exposed to oxygen deteriorated (Figure 1).

In Flavor Chemistry of Wine and Other Alcoholic Beverages; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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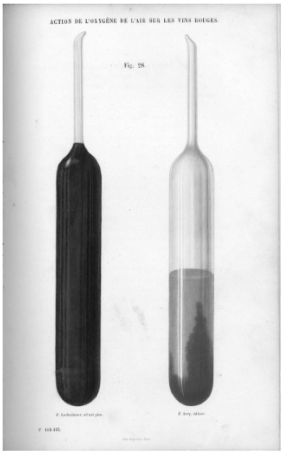
ACTION DE L'OXYGÈNE DE L'AIR SUR LES VINS ROUGES.
Fig. 28.
F. Savy, éditeur.
P. 113-115.

Figure 1. Pasteur's results wine aging without oxygen (l) and with oxygen (r) (1).

During the last 10 years there has been growing interest in the chemistry of wine oxidation, although it has been a topic of interest for a long time as well. Recent reviews on the topic inclue that by Li et al (*2*), Karbowiak et al (*3*), a review of microxygenation by Gomez Plaza at al (*4*), and my own report with Laurie (*5*). Many other studies provide good overviews of the topic as well.

It is difficult to address oxidation without some reference to a few of the major modern contributors who are no longer active. These include Ribereau-Gayon who published mostly in French, but has a short review in English (*6*) and Singleton (*7*) who developed the initial chemistry of oxidation, defining the production of quinones in the first stage of oxidation, and the oxidation of ethanol in the second. Here we will look at a few selected recent developments in wine oxidation chemistry.

The Danilewicz Papers

It would be impossible to consider the latest understanding of wine oxidation without noting the contributions and efforts of John Danilewicz. Dr. Danilewicz was a synthetic chemist at Pfizer in the UK, but on retirement he opened a vineyard near Bath, England. This appeared to stimulate an interest in wine chemistry, and he first published a review on wine oxidation in 2003 (*8*). Here he explained the background chemistry of wine oxidation from a fresh perspective. It was surprising that such a notable contribution would arrive from someone with no apparent institutional support, the correspondence from a street address in Kent! However, in this report Danilewicz outlined key redox reactions and half reaction potential that drove wine oxidation. This set the stage for his first experiments, ones that were apparently conducted in his garage adjacent to his home. However, the elegant simplicity of the studies required nothing more sophisticated than a well-calibrated burette (*9*). He showed the necessity of metals, as well as the potent accelerating effect of phenolics in oxidation reactions. Just the next year another paper came out, this time showing reactions and products of a model quinone from 4-methyl catechol (*10*).

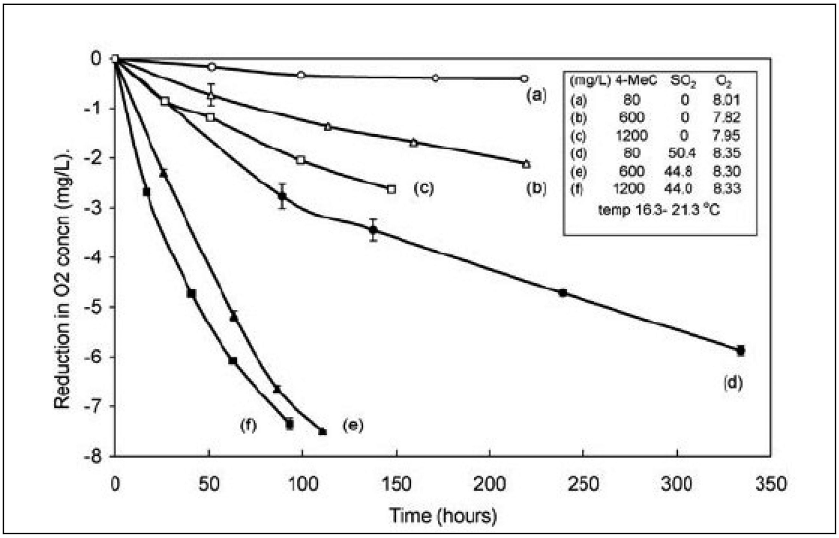

Figure 2. Increase in rate of reaction of oxygen in the presence SO_2 at increasing 4-MeC concentrations. Reproduced with permission from reference (10). © 2008 American Society of Enology and Viticulture.

The later use of a Unity INVA 600 MHz NMR suggested that the research resource base had expeanded beyond his home, with two co-authoring students from Plumpton College. Here he showed that sulfites also accelerated oxygen consumption, and the reason was that sulfites actually consumed the quinones produced (Figure 2). Without their removal, the oxidation process stalls. This demonstrated a very important point, that sulfites do not act as antioxidant by consuming oxygen, but instead by facilitating the consumption of oxygen through to the quinone, and then returning the quinone to the catechol, though some ended up as the catechol sulfonic acid. The paper so impressed members of the American Society for Enology and Viticulture that they named it their best enology paper for 2008. The next paper, published in 2010 expanded and clarified these findings (*11*). In a paper currently in press, Danilewicz studies the relative reactivity of various quinones with sulfite (*12*). So, in the space of a few years, Dr. Danilewicz has made substantial contributions using elegant studies of key questions.

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Cheynier: Flavonoid Reactions

The Cheynier lab has been contributing in a number of areas relevant to wine oxidation. This analysis will focus on their efforts in reactions between oxidation products, such as glyoxylic acid, and flavonoids, giving rise to new compounds, many of them pigmented and altering wine color. The first such study appears to be a joint effort with Joseph Vercauteren, a collaboration between Montpellier and Bordeaux, which proposed a number of possible oxidation derived dimers of catechins, linked by the formation of an electrophilic quinone, and the nucleophilic reactivity of the A-ring on the second catechin (*13*). There were a number of proposed structures because the analyses could not distinguish between a number of positional isomers. A few years later, a series of papers showed products formed by reactions of glyoxylic acid, such as this one showing a complex product likely from the addition of two molecules of glyoxylic acid, as in Figure 3 (*14*). These products often had absorbances in the visible spectrum, in this case at 450 nm, giving them the ability to change the hue of a wine.

Another significant contribution was the identification of anthocyanin adducts of oxidation products. When reported these were thought to be yeast metabolites (it is not clear what the balance of sources might be), such as the pyruvate and acetate products that made the "vitisins" as reported in 1998 (*15*). A series of related studies showed several different anthocyanin based products such as a 2002 report (*16*). Cheynier provided a short summary of this work in 2006 (*17*).

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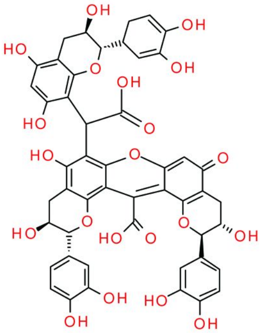

Figure 3. Product of glyoxylic acid and catechins to form new pigments (14).

Electrochemistry: Kilmartin

One of the different means to studying wine oxidation is to take the electrochemical approach. Paul Kilmartin has been leading this topic since addressing the question of the redox potential of wine in his BSc(hons). dissertation (*18*). His more recent work in the area has utilized cyclic voltammetry to analyze the ease with which compounds, wines or mixtures can be oxizided or reduced (*19*), and applying that even to analytical questions regarding the amount of free sulfur dioxide (*20*). There was one suggestion that instead of adding oxygen, an electrochemical treatment could provide the oxidation normally obtained by micro-oxygenation (*21*) although electrode fouling limited its utility. But the use of electrochemistry provides a novel view to oxidation chemistry, one that leads to new insights and may provide new means to quantify wine oxidation and antioxidants.

Clark, Scollary, and Ascorbate

The collaboration between Andrew Clark and Geoffrey Scollary, has made many contributions to wine oxidation chemistry, but their recent focus on ascorbic acids is particularly notable. Since 2008, they have published 6 papers on this topic. Ascorbate is often added to wine as a protective antioxidant, and their study has helped explain the chemistry of this action. Early work showed that ascorbate was protective of wine browning as long as it was not depleted in the wine, but when the ascorbate was exhausted, its presence enhanced browning (*22*). They then determined the structure of a catechin adduct of ascorbate, or rather its dihydro oxidation product, suggesting that it could be a pigment precursor in browning (*23*). A comparison of ascorbate versus erythorbate showed that less browning occurs with erythrobate and less of the adduct with either catechin or epicatechin (*24*). Their most recent work investigates the interaction of ascorbate and glutathione, showing that high levels of glutathione could slow browning and the consumption of ascorbate, but after prolonged oxidation, they observed the formation of glutathione catechin adducts, though such adducts were not observed in oxidized wine (*25*). Finally, this team has recently summarized the chemistry of wine and ascorbate in a review (*26*).

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Cinnamate Antioxidants

In a recent investigation of whether or not phenolics could inhibit acetaldehyde formation in a Fenton reaction, we found that in our model system they did not. This was expected because in general, the hydroxyl radical produced in the Fenton reaction, oxidizes the very first substrate it encounters, rendering a "reactive antioxidant" substance futile. But, we did observe that the amount of acetaldehyde was diminished in the presence of hydroxycinnamates, so we pursued an explanation for this effect.

Our results showed that the cinnamates were reacting with the ethanol or ethoxyl radical, and after some rearrangements and oxidation, arrived at an ethanol adduct of the cinnamate that had lost its carboxylic acid (Figure 4). This observation is the first case that this type of antioxidant reaction has been reported in food (*27*). We speculated that this product would be very reactive and likely to be dispersed into many products, but another report states that cinnamates do yield benzaldehydes analogues on oxidation (*28*). It is possible that the compounds we observed would be intermediates in the formation of the benzaldehyde products.

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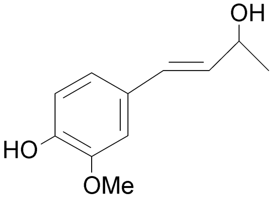

Figure 4. Hydroxycinnamate product from Fenton reaction in model wine.

Conclusions

Wine oxidation is a very active field of research today with many teams studying the question from many different perspectives. Current research is revealing the fundamental chemistry of the process, and this new information will be able to provide winemakers with an much better understanding of the process, and in turn, tools to better manage oxidation so that wine can benefit from oxidative treatments with minimal oxidative losses.

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Chapter 11

Impact of the Oxygen Exposure during Bottling and Oxygen Barrier Properties of Different Closures on Wine Quality during Post-Bottling

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This work outlines the effects of oxygen exposure during bottling and oxygen barrier properties of different closures (cork, synthetic and screw caps) on wine quality after bottling. The combination of bottling conditions and oxygen transfer rates of closures had a significant effect on the compositional and sensory properties of Sauvignon Blanc during 24 months of storage. High oxygen exposure, either at bottling and/or due to the high oxygen transfer rates of synthetic closures caused loss of freshness and fruit attributes and development oxidized aromas. Conversely, wines sealed hermetically as bottle ampoule or with closures with very low oxygen permeability such as screw caps saran-tin, are more favorable to the preservation of varietal aromas of Sauvignon Blanc wines, but also for the development of undesirable reductive compounds. Oxygen provided by cork stoppers and screw caps saranex with low OTR seems to be enough to preserve the fruit aromas and mitigate reduced and oxidized characters.

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Introduction

Wine chemical and sensory properties are extremely dependent on the amount of oxygen that wine receives throughout winemaking and ageing (*1*, *2*). Some of the opportunities for pick up of oxygen occur during transfer operations, wood barrels stage, filtration, and at bottling (*3*). During this last operation, wine can be exposed to oxygen, either during filling or/and sealing, which can result in an increase of the wine dissolved oxygen. In addition, bottling also increase the level of gaseous oxygen in bottle headspace, which varies with headspace volume and management technology (*4*, *5*). After bottling, oxygen exposure depends on the sealing effectiveness of closures, which differ on their oxygen transfer rates (OTR) (*5–9*). Synthetic closures are generally referred as offering a lower barrier to atmospheric oxygen ingress, while screw caps are almost impermeable to atmospheric oxygen, although their effectiveness depends on the permeability of the inner liners. Cork stoppers are essentially impermeable to atmospheric oxygen; however, air trapped within the cork structure can be a relevant source of oxygen that is transferred into wine during storage (*9*)

The study of the effect of the oxygen barriers properties of closures on the compositional and sensory properties of wines after bottling leads to the eternal question of whether wines require oxygen to age in bottle. This subject is controversial and has generated different opinions through time. Some authors consider that wine development after bottling is a process of reduction (*10*). In contrast, others consider that some amount of oxygen can be beneficial for wine maturation after bottling (*5*, *11*). For white wine production and storage, any exposure to oxygen, apart from oxidative juice handling is generally considered negative. This detrimental development is often related with the loss of fruit and fermentation derived flavors, and the development of oxidized characters, accompanied by an accelerated browning of color (*12*). Although it appears possible for white wines to develop in bottle in the total absence of oxygen, recent studies have suggest that reduced, struck flint, cabbage characters can develop if the wine's redox potential is too low as a result of too little oxygen exposure after bottling (*13–15*). However, some authors consider that reduced-sulfur odors are only an expression of winemaking procedures and wine chemical composition; appropriate corrective action in the winery or vineyard should eliminate the problem (*2*).

Some progress has been done in determining the reasons for the importance of oxygen, and identifying the factors that can enhance or dilute its impact, including the closure. There have been several studies assessing the influence of different closures on wine development after bottling (*13–17*). Most of them have shown that wines sealed under synthetic closures have a tendency to lose fruit attributes and develop oxidized characters over short periods of storage. In contrast, screwcaped wines scored highest for fruity aromas, maintaining the highest levels of antioxidant compounds while showing the least colour development, but also highest score for reduced characters (*13–15*). Kwiatkowski *et al.* (2007) suggested that the development of these characters after bottling is more related to the low diffusion of oxygen through closures than to the oxygen levels at bottling (*15*).

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Volatile sulfur compounds play an important role on the aroma of wines. Long chain polyfunctional thiols display a remarkable effect on the typical box-tree and tropical fruit aroma of different varietal wines, such as Sauvignon Blanc (*18*). In contrast, short-chain thiols, sulfides, disulfides, thioesters and heterocyclic compounds are often being responsible for reduced “off-flavor” characters (*19*, *20*). Hydrogen sulfide is the main volatile sulfur compound responsible for reduced “off-flavors” related with “rotten egg” and sewage like characters (*19*, *20*). This compound can be formed metabolically by yeast from inorganic sulfur compounds and sulfite, or organic sulfur compounds, cysteine and glutathione during alcoholic fermentation (*21*). However, little is known about its formation after bottling and contribution to post-bottling reductive character.

The aim of this study was to investigate the effect of the oxygen dissolved at bottling and the oxygen barrier properties of different closures on the chemical composition, color and sensory properties of a Sauvignon Blanc wine from Bordeaux region during 24 months of storage. The main purpose was to highlight the importance of the oxygen management at bottling, but also of oxygen transfer rates of closures as predictable tools for the optimization of wine chemical and sensory properties and therefore, maximize the consumer enjoyment of the product.

Materials and Methods

Wine

Wine used for the trial was produced during 2004 vintage from Sauvignon Blanc grapes grown in the Côtes de Duras (Lot-et-Garonne, France). Fermentation was carried out in stainless steel tanks under 18°C during 20 days. Tartaric precipitation was carried out in isotherm tanks under constant temperature of 3 ± 1 °C during 7 days. The chemical composition of wine before and at bottling is represented in table 1.

Bottles

Wine bottles were supplied by Saint-Gobain Glass Packaging (Cognac, France). The bottles used for cylindrical closures (cork stoppers and synthetic closures) were of Antique green colour and 750 mL of capacity, produced according to the CETIE 35-100 TR specifications. For screw cap closures, 750 mL Antique green colour bottles with a screw thread were used.

Hermetic bottles named “all-in-glass bottles” were supplied by Rudolf Gantenbrink (Limburg, Germany). These bottles were of Antique green colour and 750 mL of capacity. The airtightness of these bottles was confirmed in previous studies (*7*, *8*).

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Table 1. Sauvignon Blanc wine composition before and immediately after bottling

Compositional variable	*Value*
Measurements before bottling[1]	
Alcoholic strength	12.1 % v/v
pH	3.25
Total acidity	4.27 g/L as tartaric acid
Volatile acidity	0.29 g/L as acetic acid
Tartaric acid	1.40 g/L
Malic acid	3.02 g/L
Glucose plus fructose	0.40 g/L
Laccase activity	None detected
Acetaldehyde	42 mg/L
Iron	3.5 mg/L
Copper	0.4 mg/L
Potassium	5.2 g/L
2,4,6 – trichloroanisole, 2,3,4,6 – tetrachloroanisole, 2,3,4,5,6 – pentachloroanisole, 2,4,6 – tribromoanisole	None detected
Measurements made after bottling[2]	
Total SO_2	132 mg/L
Free SO_2	41 mg/L
Ascorbic acid	85 mg/L
Color parameters	
$A_{420\ nm}$	0.057
L*	99.29
a*	-0.70
b*	3.83
C*	3.89
h_{ab}	100.3

[1] Analysis carried out from a tank sample one day prior to bottling. [2] Analysis carried out on 3 control bottles (i.e. bottle ampoules).

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Closures

Eight sealing systems were tested in the trial: two Stelvin screw cap closures (60 mm length and 30 mm of diameter) with different liners, saran tin-foil and saranex 38, respectively; a natural cork stopper (reference “flor”, 44 mm length and 24 mm diameter), a colmated cork stopper (reference (*3*), 44 mm length and 24 mm diameter); two ‘technical’ cork stoppers, an agglomerated cork (45 mm length and 24 mm diameter) and a microagglomerate cork (44 mm length and 24 mm diameter); a synthetic co-extruded closure, (43 mm length and 22 mm diameter). A hermetic bottle ampoule sealed with glass closure tubes (40 mm length and 10 mm of diameter) as it is described in bottling and storage section was also used.

Bottling and Storage

The bottling run was initiated with the screw caps, saran-tin and saranex, respectively. The cylindrical closures were then applied in the following order: natural cork, agglomerate, colmated cork, synthetic closure and microagglomerate (Figure 1). A total of 40 bottles for each type of sealing system were sealed over a period of 2 hours. The temperature of wine during bottling varied from 11.5 to 14.2 °C

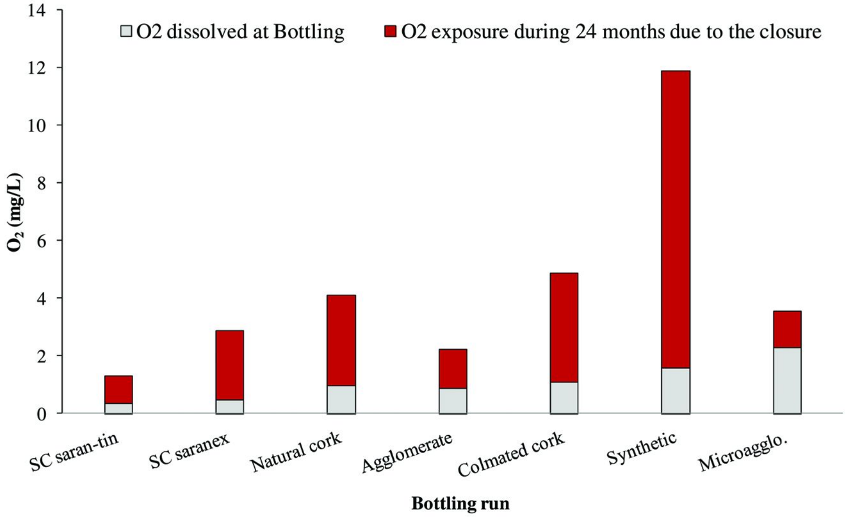

Figure 1. Oxygen dissolved at bottling and oxygen transmission through different closures during 24 months. The order of bottling is represented from left to the right. Values of oxygen during bottling are the mean of 3 bottles. Oxygen transfer rates (OTR) of closures were obtained from colorimetric measurements of 10 replicates, which were taken from the same bale of the closures used in the bottling trial. SC saran-tin = screw cap saran-tin ; SC saranex = screw cap saranex; Microagglo. = microagglomerate cork. (see color insert)

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The wine was filled into screwed bottles at 45 ± 1 mm from the top of the bottle under a cadence of 500 bottles/hour. The bottles were then sealed under a flush of nitrogen (0.1 bar), which was applied immediately prior to the insertion of screw caps. All bottles sealed with cylindrical closures were filled to 63 ± 1 mm from the top, under similar conditions described above. The cylindrical closures were compressed to a diameter of 16 mm before insertion under vacuum into bottles.

The hermetic bottles were filled directly from the filter under nitrogen flux (Glasshütte Limburg, Limburg, Germany) at 55 ± 1 mm from the top of the bottle. The bottles were then sealed with glass closures by flame welding (1200°C) to bottleneck using a sealing glass prototype (Glasshütte Limburg, Limburg Germany).

Bottles sealed with cylindrical closures were left upright for 1 hour, and then stored horizontally in stainless steel pallets. The bottles sealed with glass (bottle ampoule) and screw caps were stored vertically into cartons. All bottles were stored over 24 months under cellar conditions.

Standard Chemical Analysis

Wines were analyzed for free and total sulfur dioxide by amperometric titration corrected with acetaldehyde. Glucose, fructose, L-malic acid and acetaldehyde were determined by enzymatic assays (Boehringer, Mannheim, Germany). The pH was measured using a pH-meter CG825 (Schott-Geräte, Germany). The concentration of ethanol, titratable and volatile acidity and the concentration of tartaric acid were determined by near infrared reflectance using WineScan FT 120 (Foss France S.A., Nanterre, France). The laccase activity was measured using the enzymatic assay described by Grassin and Dubourdieu (*22*). Analysis of iron, copper and potassium were performed before bottling using Inductively Coupled Plasma Atomic Emission Spectroscopy. The concentration of ascorbic acid was determined according to the High Performance Liquid Chromatography method described by Lopes *et al.* (*23*).

The pH, volatile acidity and the concentration of ascorbic acid, free and total sulfur dioxide were also measured at 48 hours, 2, 12 and 24 months. Five replicate bottles per type of closure were analyzed at each time point after bottling.

Measurements of dissolved oxygen (3 measurements for each closure run) in wine were made using an Orbisphere 29971 (Trappes, France) sampler for bottles.

Color Measurements

The wine color was analyzed by measure of the absorbance at 420 nm using a Unikon 922 spectrophotometer (Kontron Instruments, Milan, Italy) in 10 mm quartz cuvette. In addition, wines were also submitted to Tristimulus CIELab measurements of the parameters L*(lightness/darkness), a* (red/green chromaticity), b* (yellow/blue chromaticity) and the derived values C* (chroma) and h_{ab} (hue angle) using a Minolta™ series CM – 508i spectrocolorimeter equipped with a transmittance accessory CM-A76 (Osaka, Japan). These measurements were carried out at room temperature in a 10 mm quartz cuvette using an illuminant D65 and a 10° observer angle according to the CIELab76.

GC Analysis of Different Aromatic Compounds

The 4-mercapto-4-methylpentan-2-one (4MMP) and 3-mercaptohexan-1-ol (3MH) were determined according to the method described by Tominaga *et al.* (*24*). The concentrations of hydrogen sulfide (H_2S) were determined according to the method described by Lavigne *et al.* (*25*).

The levels of sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone) were determined according to the method described by Lavigne et al. (*26*).

Five replicates of each type of closure were analyzed at 24 months of storage.

Sensory Analysis

Descriptive sensory analyses were performed at 2, 12 and 24 months post-bottling by a panel of 11 judges recruited from the staff of the Faculty of Enology of Bordeaux (France). All the assessments were performed at room temperature 18±1°C in individual booths under daylight lighting. 50 mL of wine was presented in standard ISO 3591 'XL5-type' tasting glasses with glass covers identified by three digit random codes and assessed within one hour of pouring.

The sensory attributes scored were aroma intensity, overall fruitiness and aroma freshness, reduced and oxidized characters. Wine defects were also rated, when perceived by the panelists. Panelists were instructed to assess first the aroma and then palate of wines, scoring each attribute on a scale of 0 to 5, where 0 indicated that the attribute was not perceived and 5 high intensity of the attribute. Eight samples, one per closure type, were presented to each panelist per session. At each time point, 4 sessions were carried out over two days (10 to 12 a.m.). Thus each panelist assessed 32 samples.

Data Analysis

All data were analyzed using Microsoft Excel 2000 software. Analysis of variance (ANOVA), Fisher's least significant difference, correlation and regression analyses, and PCA (principal component analysis) were carried out with XLSAT software (Addsinsoft, Paris, France).

Results and Discussion

Dissolved Oxygen at Bottling

Although most of the parameters that might have influenced the closure' subsequent performance were carefully controlled during bottling, some oxygen variations were observed due to practical constraints of bottling. The dissolved oxygen concentration in wine ranged from 0.19 to 2.4 mg/L throughout the bottling (Figure 1). The bottling run was stopped after screw caps insertion in order to change the type of bottles and to do the necessary bottling line adjustments required by cylindrical closures. Thus the level of dissolved oxygen increased significantly throughout the bottling run as the level of wine in the tank decreased and no additional protection with inert gases was done. The impact of

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the variation of dissolved oxygen at bottling on the compositional and sensory of Sauvignon Blanc wine is presented below.

Ascorbic Acid

Ascorbic acid is a powerful oxygen scavenger, which was purposely added to wines to give it an extra protection against oxidation and enhance its shelf-life (*27*). The effect of oxygen management at bottling on the levels of ascorbic acid was observed within the first months after bottling. Forty eight hours after bottling, the levels of ascorbic acid were similar in all wines, with the exception of the wine in the bottle ampoule, which presented more 6 to 7 mg/L of ascorbic than those sealed with other closures (Figure 2a). This difference was related to the bottling procedure, as wine under bottle ampoule was filled directly from the filter under nitrogen, preventing the oxygen exposure that the other wines were submitted during the regular bottling operation (filling and sealing).

During the first 2 months of storage, the concentration of ascorbic acid decreased significantly, being statistically different among bottles sealed with different closure technologies ($p < 0.001$). Under hermetic conditions (bottle ampoule), the ascorbic acid only dropped 2 mg/L, while those sealed with natural cork and screw cap lost 28 and to 27 mg/L, respectively. Losses of 33 and 39 mg/L of ascorbic acid were observed in wines sealed under agglomerate, respectively. The highest losses of ascorbic acid were observed in wines sealed with synthetic closure and microagglomerate, 46 and 57 mg/L, respectively. This result was probably related to the greater amount of oxygen dissolved in these wines at bottling when compared to the other wines (Figure 1).

The loss of ascorbic acid mainly occurred in the first two months of storage, even though after this period all wines continued to lose ascorbic acid but at different rates. In bottle ampoules and sealed with screw caps, colmated and microagglomerate corks the rate of ascorbic acid losses from two months onwards was similar, although the absolute concentrations were different. The rate of loss of ascorbic acid in wines sealed with natural and agglomerate cork was slightly higher than the precedent wines, but significantly lower than those sealed under synthetic closure. Under anaerobic conditions (i.e. bottle ampoule), almost all ascorbic acid added was retained, which clearly shows that the depletion of ascorbic acid in wines only occurs due to oxidative reactions.

A theoretical maximum consumption of ascorbic acid by oxygen can be calculated assuming a direct reaction, where 1 mole of oxygen consumes ~1 mole of ascorbic acid (*28*). Assuming this relationship, the estimated loss of ascorbic acid in wines due to oxygen dissolved at bottling and transmitted through closures is substantially lower than those really observed after 24 months. However, if the consumption of ascorbic acid due to the estimated volume of oxygen in the headspace is included, the total estimated loss of ascorbic acid in wines sealed with screw caps saran and saranex would be 37 and 45 mg/L, levels closer to those effectively observed. The theoretical losses of ascorbic acid for cylindrical closures are still lower than those observed. This seems to indicate that the oxygen amount in the headspace was underestimated, once the loss of ascorbic acid from 2 months onwards is closer to the observed values. The oxygen in

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the headspace after bottling was not determined; however, it is recognized that 50 to 65% of the total oxygen in a bottle (dissolved oxygen+gaseous headpace) after bottling resides in the headspace (*4*, *5*). In addition, the level of dissolved oxygen might have also been underestimated as the technique only measured the remaining molecular oxygen in wine, which not includes the portion of oxygen that had immediately begun to react with various wine susbtractes (e.g. metal ions, phenolic compounds, etc).

Sulfur Dioxide

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Forty eight hours after bottling, the concentrations of free sulfur dioxide in wines were identical among the different cylindrical closures (Figure 2 b). Likewise, the levels of total sulfur dioxide were very similar, being slightly lower in wines sealed under synthetic and microagglomerate closures (Figure 2 c). Under bottle ampoules, wines presented levels of free and total sulfur dioxide 5 and 20 mg/L higher than those sealed with other closures. Again, this difference was likely related with the bottling, once bottle ampoules were filled directly from the filter.

During the first 2 months of storage, the level of free and total sulfur dioxide in ampoule decreased by to 12 and 3 mg/L, respectively. Losses of 5 and 13 mg/L of free and total sulfur dioxide were observed in wines sealed under both types of screw caps; while in those sealed under natural, agglomerate and colmated corks lost 16 and 3 to 5 mg/L of free and total sulfur dioxide, respectively. The highest reductions of free and total sulfur dioxide were detected in wines sealed with microagglomerate corks and synthetic closures (Figure 2 b and c). The impact of bottling appears to be very significant once the levels of free and total sulfur dioxide decreased significantly at this stage, being more important in those bottles sealed with synthetic closures and microagglomerate corks, which contained the highest levels of dissolved oxygen at bottling.

The decrease on sulfur dioxide levels mainly occurred in the first two months of storage, even though after this period all wines continued to lose free and total sulfur dioxide but at different rates. In screw caps and colmated closures the rate of free and total sulfur dioxide losses from two months onwards was 2 to 3 mg/L and 5 and 8 mg/L, respectively. The rate of loss in wines sealed with natural, microagglomerate and agglomerate cork was slightly higher, around 5 mg/L of free of sulfur dioxide. Wines sealed natural corks lost 14 mg/L of total sulfur dioxide, while microagglomerate and agglomerate only decreased by 5 and 8 mg/L, respectively. The highest losses of free and total sulfur dioxide were found in wines sealed under synthetic closure, 10 and 24 mg/L, respectively.

The results of sulfur dioxide presented a similar trend to that found with ascorbic acid. In the first two months of storage, the levels of sulfur dioxide strongly decreased due to the oxygen introduced at bottling and then continue to drop in the 22 months thereafter, mainly in wines sealed with synthetic closure, which allows continuous oxygen entering into bottles at high rates.

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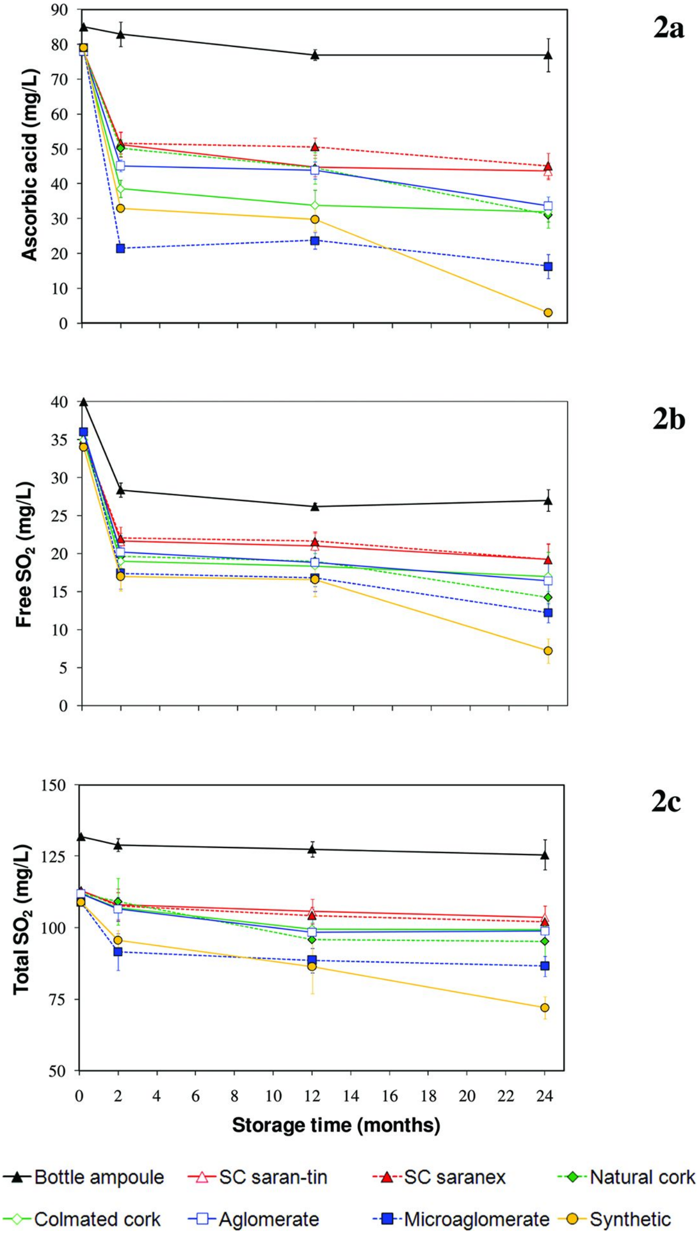

Figure 2. Impact of storage time and closure on ascorbic acid (a), free (b) and total SO_2 (c) concentrations in Sauvignon Blanc wine. Reproduced with permission from ref. (37). © 2009 American Chemical Society. (see color insert)

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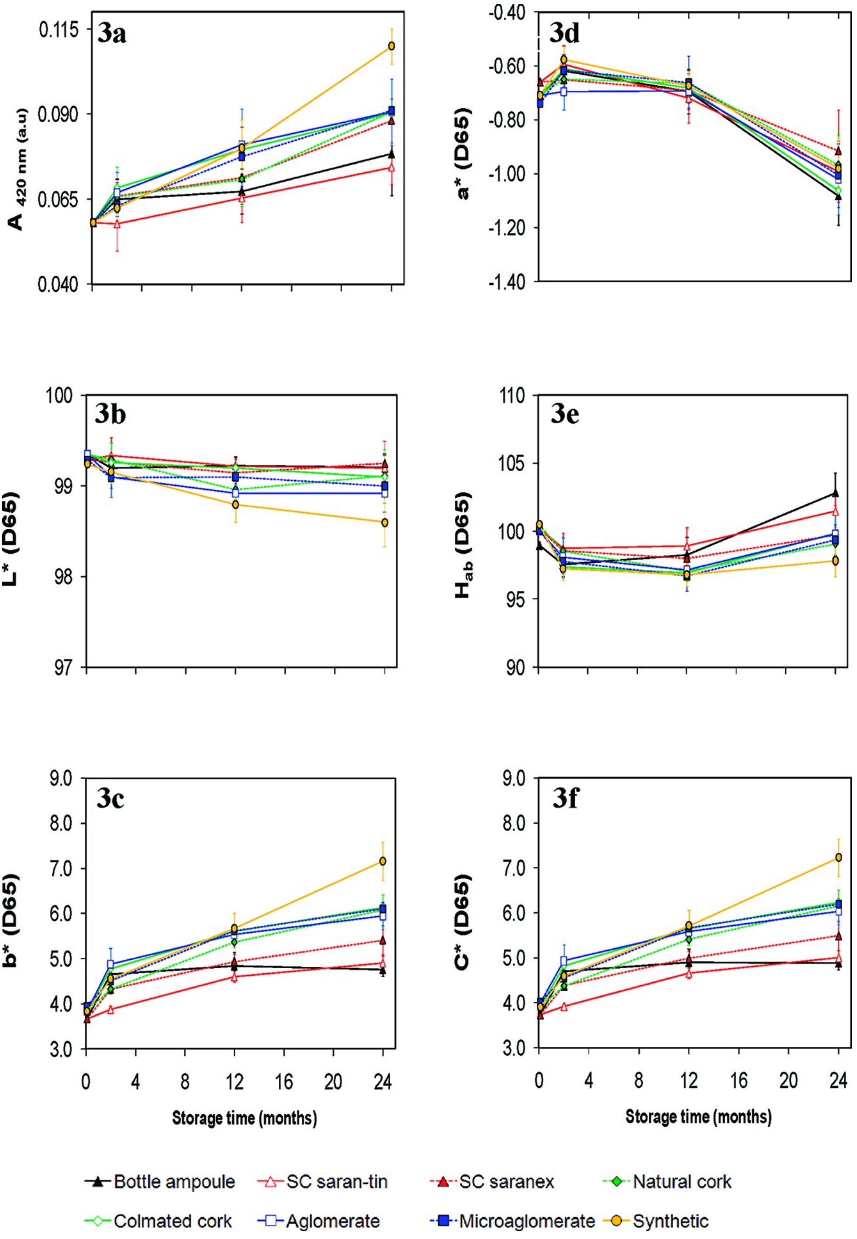

Figure 3. Impact of storage time and closure on $A_{420\ nm}$ (a) and CIELAB colour values of a Sauvignon Blanc wine: b) L(lightness/darkness), c) b* (yellow/blue chromaticity), d) a* (red/green chromaticity), e) h_{ab} (hue angle), f) C*(chroma). Reproduced with permission from ref. (37). © 2009 American Chemical Society. (see color insert)*

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Table 2. Concentrations of some volatile compounds in Sauvignon Blanc wine sealed with different closures after 24 months of storage

Compounds	*Bottling*	*After 24 months*							
		Ampoule	*Screw cap saran-tin*	*Screw cap saranex*	*Natural cork*	*Colmated cork*	*Agglomerate cork*	*Microagglomerate cork*	*Synthetic closure*
4MMP (ng/L)	n.a.	19.3 (4.4)	15.1 (6.5)	5.8 (2.9)	14.3 (0.9)	17.3 (10.4)	15.5 (2.1)	6.6 (4.6)	5.1 (1.2)
3MH (ng/L)	n.a.	821 (110)	647 (138)	396 (68)	454 (14)	361 (146)	599 (255)	436 (132)	114 (41)
H_2S (µg/L)	1.4	29.6 (4.7)	21.1 (3.6)	15.0 (3.7)	6.9 (3.6)	6.6 (2.6)	6.5 (5.5)	2.5 (1.7)	3.5 (1.9)
Sotolon (µg/L)	n.a.	n.d.	0.2 (0.2)	0.1 (0.0)	0.3 (0.0)	0.6 (0.6)	0.3 (0.3)	0.9 (0.4)	1.1 (0.6)

The name letters in the same row indicate no significant difference between the corresponding values ($p = 0.05$). n.d. : below detection limit n.a.: non-analysed Standard deviations of 5 replicates are given in parentheses

Color Measurements

$A_{420\ nm}$

The values of $A_{420\ \mathrm{nm}}$ for the different wines during the 24 months of storage are represented in Figure 3a. The $A_{420\ \mathrm{nm}}$ values didn't change during the forty eight hours after bottling. After this period, $A_{420\ \mathrm{nm}}$ increased during time, although at 2 months the values were statistically similar across all wines ($p=0.05$). At 12 months, the $A_{420\ \mathrm{nm}}$ values in bottles sealed under synthetic, agglomerate, colmated and microagglomerate stoppers were slightly higher, but statistically significant, when compared to bottles sealed under screwcaps, natural cork and ampoule ($p=0.006$). After 24 months of storage, the differences became much more evident as the bottles sealed with synthetic closures displayed significantly higher $A_{420\ \mathrm{nm}}$ values than bottles sealed with other closures ($p<0.001$). The wines sealed under screw cap saran-tin and ampoule bottles presented the lowest $A_{420\ \mathrm{nm}}$ values ($p<0.001$).

CIELab

Wine color was also assessed using the CIELab coordinates (Figure 3bcdef). During the 24 months of storage, the wine color became more yellow (higher b* and C* values) and more intense (lower L*). The L* (lightness) values of wines were not significantly affected during storage, with the exception of wines sealed with synthetic closures, where L* values decreased significantly, mainly from 2 months of storage onwards ($p<0.001$). At 24 months, the lightest wines were sealed under ampoule and screw cap saran-tin and the darkest were sealed with synthetic closure ($p<0.001$) (Figure 3b). The a* values of wines remain stable during the 24 months of storage regardless the type of closure used to seal it ($p=0.05$) (Figure 3d). In contrast, b* and C* coordinates increased over time; the highest values were observed for wines sealed with synthetic closures, lowest for those sealed under ampoule and screw caps, and intermediate for other wines (Figure 3c and f).

The h_{ab} values decreased slightly during the first two months of storage, followed by an increase in 22 months thereafter, which mainly occurred between 12 and 24 months. At 24 months, the highest values hab were observed for wines sealed under ampoule and screw cap saran-tin and the lowest for those sealed with synthetic classic ($p<0.001$) (Figure 3e).

The ΔE^*_{ab}, a measure of color differences between samples, was also calculated (data not shown). The results obtained showed that, in general, color variations among different wines were not susceptible to be perceived by the human eye as the ΔE^*_{ab} value were lower than 1. The only exception was observed at 24 months where the ΔE^*_{ab} value, between either ampoule or screw cap saran-tin and synthetic closures were greater than 1, which confirmed the visual observations. These findings confirmed that wine color changed throughout storage, being particularly distinctive at 24 months between wines that presented the largest differences on oxygen exposure at bottling or during storage due to the

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high oxygen transfer rates (OTR) of closure and those sealed under more airtight conditions (bottle ampoule and screw caps).

Aromatic Composition

Varietal Thiols

3-mercaptohexan-1-ol (3MH) and 4-mercapto-4-methylpentan-2-one (4MMP) are key volatile thiols responsible for the distinctive varietal grapefruit, passion fruit and box tree aroma of Sauvignon Blanc wines (*18*). Therefore, these compounds should be preserved as much as possible during storage in order to keep the flavor identity of the Sauvignon Blanc wine.

The concentrations of 3MH and 4MMP after 24 months in bottle are represented in table 2. The levels of these compounds were well above the perception thresholds in wines, 60 ng/L for 3MH and 0.8 ng/L for 4MMP (*18*, *24*). However, wines sealed with synthetic closures presented the lowest concentrations of both of these compounds, which levels were barely above their perception thresholds in wines. The highest concentrations of 4MMP were found in wines sealed under bottle ampoule, but without significant differences to those sealed with screw cap saran-tin, natural, colmated and agglomerated stoppers ($p=0.05$). Wines sealed with screw cap saranex and microagglomerate cork presented lower levels 4MMP, which were not significant different from those observed in wine sealed with synthetic closures. The highest concentrations of 3MH were found for wines sealed under bottle ampoule followed for those sealed with screw cap saran-tin and agglomerated stoppers, the lowest for those sealed with synthetic closures and intermediate for the other wines. These findings have shown that both thiols are oxygen sensitive once their lowest concentrations were detected in wines that were more developed due to the high exposure to oxygen either during bottling or during the storage due to high oxygen transfer rates of closures (*29*). The precise mechanisms by which the varietal thiols can be oxidized it remain unknown. However, it possible that under oxidative conditions, oxidized electrophilic phenolic compounds such as quinones, can react with thiols (3MH and 4MMP) to form flavor less compounds and consequently lead to an loss of the varietal characters of Sauvignon Blanc wines (*30*).

Surprisingly, the concentrations of 3MH and particularly 4MMP were relatively low in wines sealed with screw cap saranex; although, the levels of ascorbic acid and sulfur dioxide, and color parameters did not indicate that oxidation level was more pronounced than wines sealed with screw cap saran-tin and cork stoppers. This observation suggests that these compounds can be lost due to non-oxidative mechanisms, such as scalping. Recent studies suggest that flavor scalping is one of the main mechanism by which wines sealed under Tetrapack and "bag-in-box" loss its nonpolar flavors compounds (*31*, *32*). In addition, closures also display different sportive capacities, which are more marked with synthetic closures than with natural corks and screw caps (*31*, *32*). The screw cap liners are formed by the assemblage of different polymer layers; while screw cap saran-tin is composed by polyethylene, kraft, tin and PVDC, the saranex liner is essentially composed of PVDC and polyethylene with this last

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polymer being in contact with wine. The polyethylene is well known to remove volatile compounds through flavor scalping and therefore can have contributed for the larger thiols losses of screw cap saranex compared with screw cap saran-tin sealed wines (*33*). The fact that 4-MMP and 3-MH behaved differently from the hydrogen sulfide (see below) can somehow support the scalping hypothesis.

Hydrogen Sulphide

The concentration of hydrogen sulfide (H_2S) was determined at bottling and after 24 months of storage (table 2). Immediately after bottling, the concentration of H_2S was 1.4 µg/L, which is close its sensory threshold in wines. After 24 months of storage, the concentrations of H_2S were highest in bottle ampoule and in screw cap sealed wines; while those sealed under cork stoppers and synthetic closure had the lowest levels of H_2S. Although, the H_2S level increased throughout storage for all wines, it was far much more prevalent in wines sealed under hermetic conditions and under very low oxygen transfer rates closures, such as screw caps. This is consistent with the previous findings showing that reductive characters such as struck flint, rubber, rotten eggs aromas were far more prevalent in screw caps and ampoule sealed wines (*13–15*).

The reactions behind the formation of this compound after bottling are not totally understood; however, it seems likely that H_2S can be formed from the reduction of sulfate or sulfite catalyzed by transition metals (iron or copper), phenols or ascorbic acid, when oxygen levels in bottle are near nil (*21*, *34*, *35*). Then, the H_2S could either accumulate in wines under anaerobic conditions or be readily oxidized when in contact with oxygen introduced at bottling or permeating through the closure.

Sotolon

Sotolon (3-hydroxy-4,5-dimethyl-2(5)H-furanone) is a volatile compound with an intense odor of *curry* and *rancio* that could contribute to the oxidation aromas of prematurely aged dry white wines. At 24 months, the results obtained showed that are all wines presented concentrations of sotolon below its wine perception threshold, 2 µg/L (table 2). Nevertheless, it was interesting to observe that under hermetic sealing as bottle ampoule, this compound was not detected; while, wines highly exposed to oxygen either at bottling or due to the high closure OTR exhibited the highest concentration of sotolon. These findings seems to be consistent with recent evidences that the formation of sotolon after bottling is related with the ability of closures to exclude oxygen, being highest in wines sealed with synthetic closures when compared to those sealed under cork stoppers (*26*).

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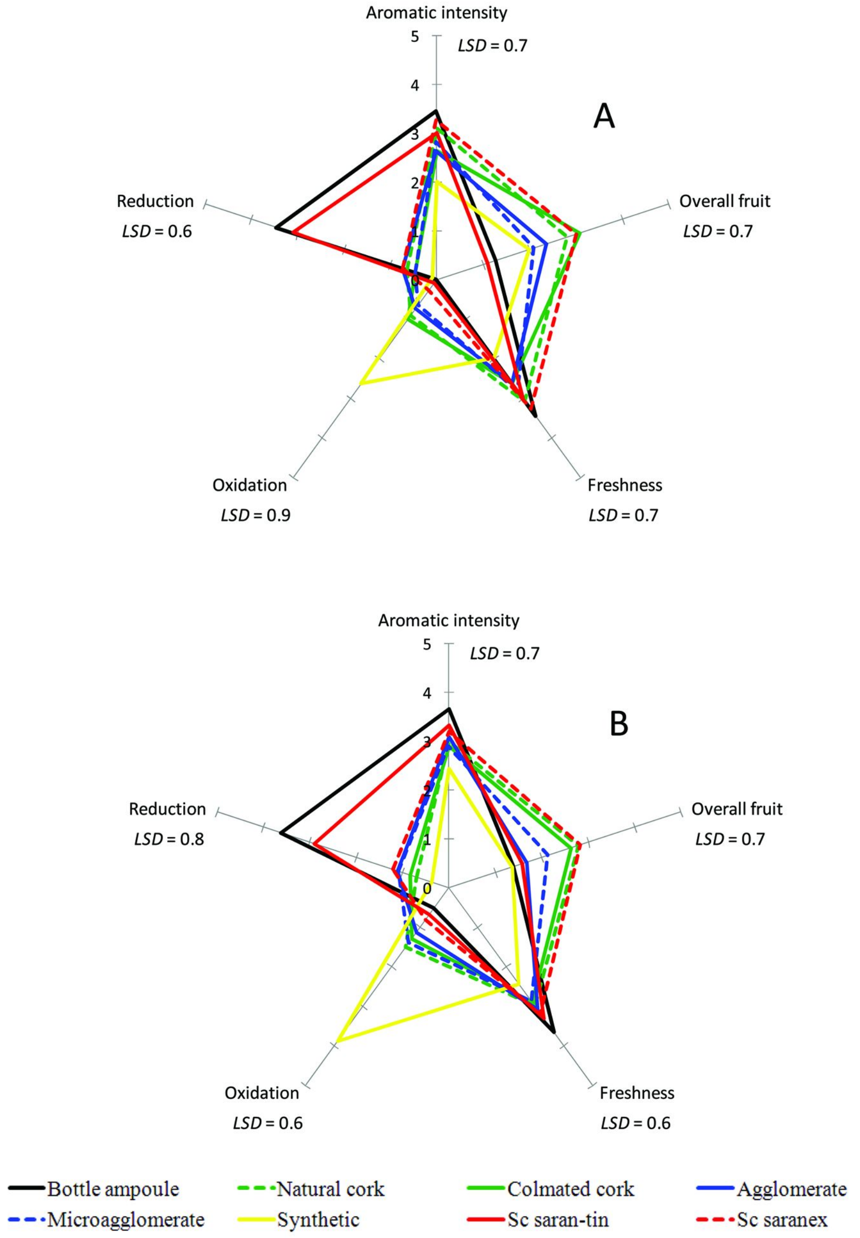

Figure 4. The effect of closure treatment on selected sensory attributes for a Sauvignon Blanc wine after a) 12 months, b) 24 months of storage. Values at 12 and 24 months are the means of 4 replicates. Least significant differences (LSD) at the 5% level are indicated. Reproduced with permission from ref. (37). © 2009 American Chemical Society. (see color insert)

Sensory Analyses

The results of the descriptive analysis carried out at 2 months showed that differences between the closures were not statistically significant (data not shown). In contrast, after 12 months of storage, significant differences among the closures samples were detected for each of the attributes assessed (Figure 4a). The synthetic closure was distinctly differentiated from the other closures, displaying the highest score on oxidation and the lowest in aromatic intensity and freshness. Conversely, ampoule and screw cap saran-tin samples presented the highest scores in reduction and lowest in overall fruit and oxidized characters ($p<0.001$ and $p=0.007$). The microagglomerate and agglomerate sealed wines were rated significantly lower in overall fruit than those sealed with natural and colmated corks, and screw cap saranex, which were rated as the highest in this attribute ($p<0.001$).

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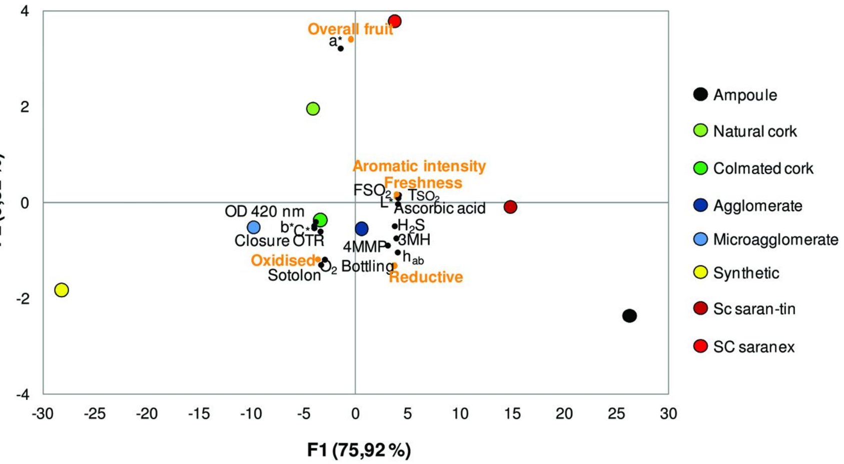

Figure 5. Biplot of principal components analysis of the sensory and compositional attributes of a Sauvignon Blanc bottled wine sealed under 8 different sealing systems for 24 months of storage. The 8 wines are represented as larger symbols with the sensory and compositional variables represented by small orange and blue small circles, respectively. Compositional attributes: 3MH = 3-mercaptohexan-1-ol; 4MMP = 4-mercapto-4-methylpentan-2-one; H_2S = hydrogen sulfide; $[O_2]$ bottling = oxygen dissolved at bottling; Closure OTR = oxygen transfer rates. (see color insert)

After 24 months of storage, the sensory differences become more pronounced than those observed at 12 months. Wines sealed under ampoule and screw cap saran-tin were rated highest in reduced characters compared to the other closures. Wines sealed with synthetic closures were rated highest in oxidation, which negatively affected the aroma intensity, freshness and overall fruit attributes. For overall fruit character, the wines sealed under colmated, natural corks and screw cap saranex receive the highest rates, those sealed under microagglomerate cork rated the intermediary and the other wines were rated with lowest scores (Figure 4b). The sensory analysis confirmed the compositional and color analyses and their relationships are represented below (Figure 5).

Principal Components and Correlation Analyses

Figure 5 present the 24 month sensory and compositional analysis results for 8 different closures technologies in this trial on a principal component analysis (PCA). This technique facilitates the visualization of the differences and similarities between wines sealed under different closures. Wine compositional parameters and sensory attributes at 24 months, oxygen content at bottling and oxygen transfer rates of closures variables that display a strong relationship with each other are clustered close together in Figure 5. The wines plotted far from the origin were highest in those variables situated in close proximity.

The results clearly show that a poor oxygen management at bottling and bottling with different closures generated a Sauvignon Blanc wine with different compositional and sensory properties after 24 months of storage. The bottle ampoule and screw cap saran-tin were primarily separated by its high concentration of antioxidant (ascorbic acid and sulfur dioxide), low color development and highest in 3MH, 4MMP. These wines were also rated high in freshness and aromatic intensity, but also in reductive characters which was associated with high levels of H_2S. The wines rated with the highest fruit intensity developed under natural cork but also with screw cap saranex, which was able to mitigate reduced like aromas, i.e. levels of H_2S presented by these in wines were not high enough to spoil the wine. In contrast, wines with oxidized characters developed under synthetic closures situated in the bottom left quadrant, where the wines presented the highest OD 420 nm, b*, c* and sotolon concentration. The microagglomerate cork was further discriminated on the basis of its oxygen content at bottling and sotolon level after 24 months. Both agglomerate and colmated cork were close to the origin, presenting intermediary chemical composition and balanced sensory attributes.

The Sauvignon Blanc wine style evolution is consistent with the different oxygen content at bottling, but also with the different oxygen transfer rates of closures. Wines displaying the highest oxidized characters, high color development (high OD 420 nm, C*, b*) and high concentration of sotolon are consistent with those sealed either submitted to high oxygenation at bottling and/or those sealed under closures with high oxygen transfer rates (OTR). Closures with low OTR such as natural cork, colmated and screw cap saranex generated high fresh fruity wines with a relatively balanced concentration of varietal thiols, antioxidant compounds and color development. Under hermetic conditions or with very low OTR, wines presented high levels of H_2S, which were responsible for the strong reductive, “rotten egg” and “putrefaction” characters detected in wines sealed in bottle ampoule and screw cap saran-tin.

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This study, together, with the results of previous research, indicate that the combination of bottling conditions and oxygen transfer rates of different closures have a significant effect on compositional and sensory properties of wines during post-bottling. The different style evolution generated by different closures was significant and probably strong enough to have an impact on the consumer's liking of this wine. The fact that consumers can react negatively to the presence of TCA, but also to oxidation and reduction, emphasizes the importance of the oxygen management at bottling, and oxygen barrier properties of closures that can optimize wine sensory properties and therefore, maximize the consumer enjoyment of the product (*36*). Thus, the oxygen management at bottling and the choice of wine closure type are key variables to be taking into account during winemaking and post-bottling.

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Chapter 12

Fermentation and Post-Fermentation Factors Affecting Odor-Active Sulfur Compounds during Wine Bottle Storage

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The influence of different winemaking variables on the evolution of volatile sulfur compounds during wine storage in the bottle was investigated. Addition of nitrogen to Shiraz grape must in the form of diammonium phosphate resulted in wines developing increased dimethyl sulfide concentration in the bottle when nitrogen was increased from 100 mg/L to 400 mg/L. Presence of glutathione at bottling at a concentration of 20 mg/L resulted in wines with increased H_2S after six months in the bottle. Higher exposure to oxygen during bottle storage was detrimental to the preservation of the fruity aroma compound 3-mercaptohexanol, although it also decreased the concentration of the off-odor compounds H_2S and methyl mercaptan.

Introduction

Volatile sulfur compounds (VSCs) play a key role in defining the aroma characteristics and quality of alcoholic beverages (*1*). This large family of aroma compounds includes chemicals with very different chemical and sensory properties, which, in the case of wine, can contribute either favorably or negatively to the final aroma composition of the product.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

‘Reduction’ is a term often used to describe wines exhibiting aroma properties reminiscent of rotten egg, cabbage, garlic, putrefaction. These aroma attributes, which are generally considered to contribute negatively to overall sensory quality, have been associated with the occurrence of different low molecular weight VSC, including H_2S, methyl mercaptan (MeSH), ethyl mercaptan (EtSH), and dimethyl sulfide (DMS). Because of their association with the aroma descriptors that are linked to ‘reduction’, in the context of wine these VSCs are often referred to as ‘reductive’. The origin of reductive VSCs in wine is complex, and their occurrence and concentration depends on multiple factors. Generally speaking, in the life of a wine it is possible to distinguish certain phases where reduction occurs to a greater extent (Table 1). Yeast fermentation is frequently associated with the occurrence of reductive off-odors. The microbiological and metabolic factors responsible for the occurrence of reductive odors during this phase of winemaking have been previously described (Reviewed in ref. (*2*)). From a chemical point of view, this stage of reduction, characterized by a clear rotten egg odor, is mainly linked to the formation of H_2S by the yeast. Mercaptans such as MeSH can be formed by yeast metabolism, but to date there is little evidence for this in the case of wine fermentation. Due to a number of factors, including yeast genetic background, grape composition and fermentation conditions, the amount of H_2S produced by yeast during fermentation varies to an extremely large extent, so that some fermentations can display very intense reduction characters, while others are essentially ‘clean’. As nitrogen availability is considered one of the main modulating factors for H_2S production by yeast, one commonly adopted strategy is to supplement fermentation with easily assimilable nitrogen such as ammonium salts (*3*). Although from a sensory point of view the ‘reduction’ perceived during fermentation can be very intense, most wines at the end of fermentation exhibit low H_2S concentrations, and consequently low levels of perceived ‘reduction’. Anecdotal evidence indicates that, during further processing and storage of wines in the cellar, for example during tank or barrel maturation with or without lees, reductive characters might reoccur and require specific intervention to be eliminated. Generally speaking, however, under the condition commonly adopted in the modern wine industry, most wines are bottled without any sensorially detectable reductive off-odor. However, it has been long known that, during its storage in bottle, wine can develop reductive aroma characters again, which, from a sensory point of view, appear to be more complex, with descriptors ranging from struck flint, to cabbage, to rotten egg. This second stage of formation of reductive aromas is of particular concern for winemakers, as it occurs in the finished product that is delivered to consumers (*4*). Wine maturation in the bottle is essentially a micro-aerobic process, and little oxygen availability is thought to promote the accumulation of some of the VSC associated with reductive off-odors (*5*, *6*). Nevertheless, even under highly anaerobic conditions, the occurrence of reductive odors is not systematic, which highlights the fact that certain wines have a propensity to develop reductive off-odors during bottle storage (*7*).

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Table 1. VSCs mainly involved with reductive off-odors during different stages of the winemaking process

Phase of winemaking	*Chemical compound(s) involved*	*Influencing factors*
Fermentation	H_2S	Yeast, available nitrogen, must turbidity, presence of elemental sulfur
	Mercaptans	Yeast strain, available nitrogen, others unknown
Post-fermentation	H_2S and mercaptans	Storage on yeast lees, degree of oxygen exposure
Post-bottling	H_2S and mercaptans	Presence of appropriate precursors; presence of natural antioxidants (e.g. glutathione), oxygen exposure, others not known
	DMS	Concentration of precursors (S-methyl metionine); storage temperature

Other classes of VSCs are present in wine, which are not linked to reductive off-odors. For example, polyfunctional thiols are key contributors to the passionfruit and grapefruit aromas that are characteristics of certain white wines (e.g. Sauvignon Blanc), and have been identified also in several red wines (*8*, *9*). Powerful aroma compounds such as 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexyl acetate (3MHA), and 3-mercaptohexanol (3MH) are included in this group, the latter being typically the most abundant one. 3MH is present in grapes in the form of odorless precursor, and it is released by the yeast during alcoholic fermentation (*8*). Following fermentation, 3MH concentration typically declines, which is thought to be linked to reaction with electrophiles present in the wine environment (*10*). Oxygen exposure of the wine is known to be one major driver affecting 3MH decline after fermentation and especially during bottle maturation (*6*), although more recently it has been shown that the extent of this decline is strongly affected by wine composition (*11*).

This article presents some recent observations regarding the winemaking factors responsible for the formation and degradation of VSCs during wine bottle maturation.

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Materials and Methods

Wines

For the study on the effect of di-ammonium phosphate (DAP) on the formation of DMS during ageing of Shiraz wines, one lot of Shiraz grapes with an average yeast assimilable nitrogen (YAN) of 103 mg/L (free alpha-amino nitrogen (FAN)= 71 mg/L, ammonium=32 mg/L), 23.8° Brix, and pH 3.4 was selected. Grapes were hand-picked and collected in 20 kg crates. Once in the winery, different crates were pooled together to obtain a homogenous sample. Individual 30 kg lots were then destemmed and crushed, and the must was collected in 34 L glass containers. Potassium metabisulfite was added at 100 mg/kg to each fermentation lot. DAP additions were performed according to an experimental design consisting of three YAN concentrations, each one fermented in triplicate, for a total of nine fermentations. A control that did not received any DAP addition represented the lowest nitrogen concentration (103 mg/L YAN), while in the two additional treatments the final YAN concentration was 250 mg/L YAN and 400 mg/L YAN, respectively. The samples were inoculated with *S. cerevisiae* 796 (AEB Mauri) at a rate of 1 x 10^6 cells/mL, following rehydration in water at 40°C for 30 min. Fermentations were carried out at 22 °C, with the cap plunged three times per day. The wines were left to macerate on grape solidsuntil the slowest treatment reached dryness (residual sugars $\leq$2 g/L), after which the fermented musts were pressed, the wines collected in 20 L glass containers and placed at 4 °C under inert headspace for cold settling. No malolactic fermentation was carried out. After 4 weeks 150 mg/L of potassium metabisulfite was added to the wines, which were bottled in 375 mL bottles under ROTE closures with Saran tin wad. Samples were analyzed for DMS concentration after 2, 24, and 36 months of storage at 12 °C. Additionally, samples after 2 months of bottle storage were submitted to a model aging study. For this, wines were transferred into glass flasks, pH was adjusted to

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3.5 with either 1 M NaOH or 1 M HCl, the headspace was flushed with N_2, and the samples were stored at 30 °C for 6 weeks.

The effects of glutathione and oxygen exposure on VSCs were studied in a Sauvignon Blanc wine from the Adelaide Hills region 2008 vintage, obtained from a local winery. Analytical parameters of the wine were as follows: pH 3.4, residual sugars 3.4 g/L, alcohol 13.9%, volatile acidity 0.42 g/L (as acetic acid), titratable acidity 5.6 g/L (as tartaric acid), free SO_2 41 mg/L, total SO_2 180 mg/L, 0.1 mg/L copper. Before bottling, the wines received an addition of either no or 20 mg/L of food grade GSH (Kirkman, Lake Oswego, OR) and copper sulphate to result in a final concentration of 0.3 mg/L of copper. All wines were bottled under Nomacorc Premium co-extruded synthetic closures (Nomacorc, Zebulon, NC). Before bottling, closures were stored at 20°C for one week in either air or under nitrogen for 1 week to evaluate the effects of oxygen contained in the closure on wine development. Once bottled, the wines were stored at 20°C in either air or under nitrogen to study the effect of oxygen exposure. For the treatments requiring storage under nitrogen, closures or wines were kept in steel drums filled with nitrogen and sealed. Drums were periodically refilled with nitrogen to maintain oxygen content below 10 hPa. In total, 3 different closure/storage combinations were applied to all the wines: closures previously stored in air used to seal wines in bottles subsequently stored in air (A/A), closures previously stored in air used to seal wines in bottles subsequently stored in nitrogen (A/N), closures previously stored in nitrogen used to seal wines in bottles subsequently stored in nitrogen (N/N). For the bottling of each wine, empty 375 mL flint glass bottles were flushed with 98% N_2 and then filled using a Framax FCS 4/1S automatic filling machine (Framax, Serravalle Pistoiese, Italy). Closures for different treatments were then applied with a Bertolaso Epsilon R corker (Bertolaso, Zimella, Italy) with the vacuum set at -15 kPa. A bottle fitted with two PreSens Pst3 oxygen sensors (Presens, Regensurg, Germany), to measure dissolved and headspace oxygen, was filled with wine and sealed after approximately every ten bottles in order to monitor performance across the whole bottling operation: five 'PreSens' bottles in total were filled for each wine and closure/storage combination. All oxygen measures were carried out using a PreSens Fibox 3 trace v3 oxygen meter (Presens, Regensurg, Germany). Limit of quantification of oxygen for this method was 0.2 mg/L. Generally, dissolved oxygen values, measured 24 h after bottling, were never higher than 1.12 mg/L and headspace oxygen always below 0.95 mg/L. For each 375 mL bottle, total oxygen pickup during bottling operations was between 1.32 and 1.95 mg/L.

A separate experiment was carried out to measure the amount of oxygen entering in the bottles under the three different storage conditions of this study. For this purpose, bottles of the same type described above, fitted with PreSens Pst6 oxygen sensors for measures of trace oxygen levels, were placed in a corking machine and flushed with a stream of 98% N_2 to obtain an oxygen pressure lower than 0.5 hPa. Once this oxygen level was achieved, the N_2 line was removed from the bottle neck, and the bottle was immediately sealed with Nomacorc Premium closures previously equilibrated in either air or nitrogen, as described above. One hour after insertion of the closure the oxygen pressure was measured, and then the bottles were stored in air or nitrogen, as described above. Five replicates were used for each condition (A/A, A/N, N/N). Measures of oxygen pressure were taken every 24 hours during the first week, then once a week for the following four weeks, then at three and six months of storage. For each condition, the measures allowed quantification of the amount of oxygen released from the closure at bottling, as well as of the theoretical amount of oxygen entering through the closure. After 6 months of bottle storage, the degree of oxygen exposure of the wines in the bottles, expressed as oxygen ingress in the bottle under the three experimental conditions of the study, was as follows: A/A=4.1 mg/L, A/N=3.1 mg/L, N/N=1.4 mg/L.

Analytical Procedures

H_2S, MeSH, and DMS were determined by static headspace analysis with gas chromatography-atomic emission detection (GC-AED), as described elsewhere (*12*). 3MH was analyzed by means of gas chromatography-mass spectrometry using a stable isotope dilution assay (*11*). Free amino nitrogen (FAN) was measured as described in ref. (*3*).

Results and Discussion

Influence of DAP Addition on DMS Formation during Aging of Shiraz

Figure 1 shows DMS concentration at 2 months after bottling, after the model ageing experiment and after three years of bottle ageing of the Shiraz wines. Addition of DAP did not affect DMS initial concentration. DSM concentrations at this stage were generally low, consistent with the well accepted view that DMS is a minor aroma contributor of young wines. Conversely, during both model aging and cellar aging, wines obtained from fermentations having an initial YAN of 400 mg/L (by DAP addition) showed increased final concentrations of DMS. The majority of studies on aged red wine aroma indicate that DMS can be a major contributor to the aging 'bouquet' of wine. In particular, in the case of red wine, it has been shown that, depending on concentration, DMS can contribute to both red fruit/black currant or truffle and black olives aromas (*13*). The data reported herein suggest that addition of DAP before fermentation to increase YAN can result in higher DMS fermentation after a period of ageing. This was consistently observed in the accelerated aging study as well as under typical conditions of bottle storage. Formation of DMS during wine ageing has been linked to degradation of the precursor *S*-methylmethionine (*14*), although enhanced formation of DMS during ageing has also been observed in conjuction with higher levels of cysteine (*15*). Although we did not measure the concentration of specific DMS precursors, analysis of residual free amino nitrogen (FAN) indicated that wines from fermentation supplemented with DAP to achieve either 250 mg/L or 400 mg/L YAN had respectively 17±2 mg/L and 34±3 mg/L of FAN more than wines from non supplemented fermentations. This appears to support the hypothesis that the increase in DMS observed during aging of wines from high DAP fermentations could be linked to higher concentrations of amino acid metabolism derivatives. Other authors have also hypothesised that yeast can

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form DMS precursors (*16*), but the relevance of this possible pathway in wine fermentations is not known. The data of this study indicate that fermentation practices such as YAN management, although generally aimed at influencing yeast performance and yeast-derived aroma compounds, can affect formation of aroma compounds during wine aging. It is worth mentioning that only wines from 400 YAN fermentations exhibited higher DMS concentrations after aging, and that the initial grapes had very low YAN. Similar studies should be carried on a larger range of initial grapes and nitrogen additions in order to rationalize the effect of fermentation nitrogen management on DMS evolution during wine aging.

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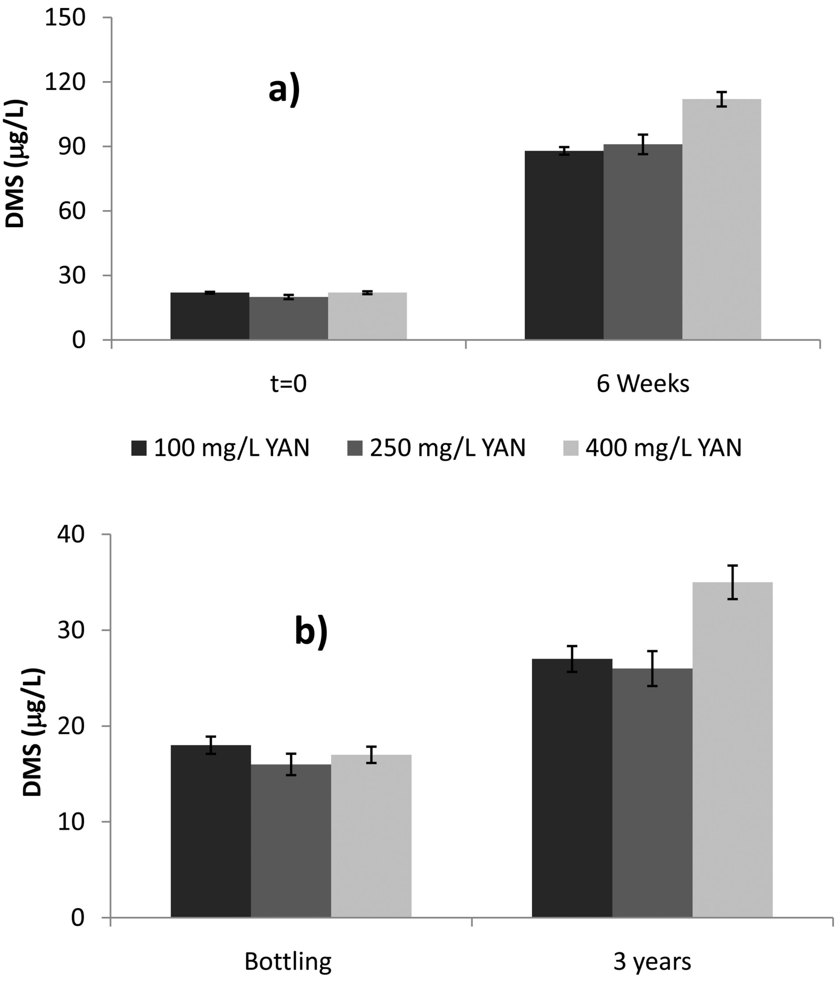

Figure 1. Influence of nitrogen addition before fermentation on the formation of DMS during a) accelerated aging and b) bottle aging of Shiraz wines.

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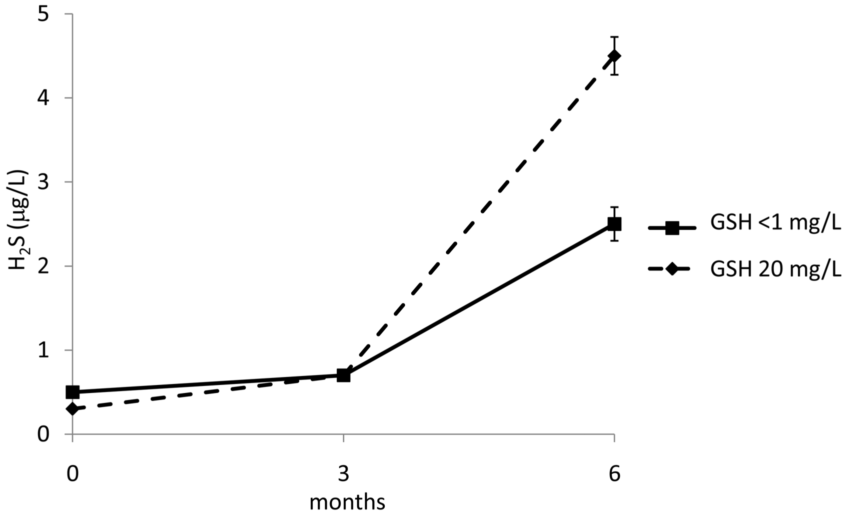

Figure 2. Influence of glutathioneconcentration at bottling on the formation of H_2S during bottle storage of Sauvignon Blanc wines in inert atmosphere.

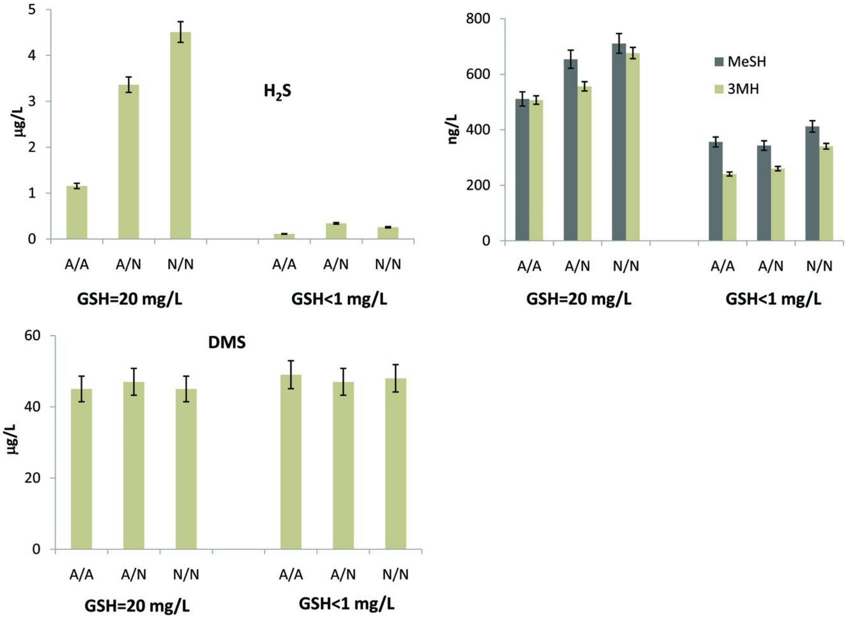

Figure 3. Effect of post-bottling oxygen exposure on the concentration of H_2S, MeSH, 3MH and DMS in Sauvignon blanc wines after six months of bottle aging. Oxygen ingress in the bottles for the different treatments was as follows: A/A = 4.1 mg/L; A/N = 3.1 mg/L; N/N = 1.4 mg/L.

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Influence of Glutathione on the Evolution of H_2S during Bottle Aging of Sauvignon Blanc

Figure 2 shows the evolution of H_2S during bottle aging of Sauvignon Blanc wines with minimal oxygen exposure (bottles stored in nitrogen atmosphere). After 6 months of storage, wines with higher GSH showed a nearly two-fold increase in H_2S concentrations, while no difference was observed after three months in the bottle. H_2S is one of the main aroma compounds contributing to the reductive character of wines, its odor threshold in white wine being 1.6 μg/L (*17*). Large quantities of H_2S are often produced by yeast during fermentation, although there is no proven correlation between the amount of H_2S produced during fermentation and the final H_2S concentration in the wine (*18*). On the other hand, it has been shown that H_2S can increase during bottle aging, so that wines that are bottled with sensorially undetectable concentrations of H_2S can develop reductive characters during bottle aging, due to accumulation of H_2S (*6*). The data in Figure 3 indicate that this process of accumulation is enhanced by glutathione (GSH) concentration at bottling. Glutathione is a naturally occurring antioxidant present in grapes, its concentration in the finished wines being affected by several factors, including grape content, degree of oxygen exposure during must preparation, and yeast strain (*19*). During fermentation, GSH is initially consumed by the yeast, to be then released towards the end of fermentation and during lees contact (*19*, *20*). Because the thiol group of GSH is highly reactive towards key oxidation compounds such as quinones, GSH is thought to have a protective action against wine premature aging (*19*). In particular, it has been shown that GSH can effectively reduce the loss of certain thiol compounds responsible for the pleasant fruity aromas of wines such as Sauvignon Blanc. However, the data in Figure 2 indicate that other –SH compounds such as H_2S, which are generally regarded as negative for wine aroma quality, are also positively affected by higher GSH concentrations.

Due to the action of GSH on thiol compounds having both positive or detrimental aroma characteristics, the sensory implications of GSH concentrations at bottling on wine aroma evolution require more detailed investigation. Likewise, the chemical mechanisms responsible for higher H_2S concentrations in wines containing more GSH remain to be determined. These might involve direct generation of H_2S from GSH, or alternatively formation of H_2S from other precursors, with GSH protecting the newly formed H_2S from the attack of wine electrophiles, for example quinones.

Effect of Post-Bottling Oxygen Exposure on H_2S, MeSH, DMS, and 3MH

Understanding the importance of oxygen to the aroma quality of wines is becoming a major area of interest for the wine industry (*4*). In particular, it has been shown that oxygen exposure in the bottle, as obtained by the use of closures having different permeabilities to oxygen, can affect wine sensory quality and consumers' preference, which seems to be closely linked to the effect of oxygen exposure on wine VSCs (*4*, *6*). Figure 3 shows the effect of increasing degrees of oxygen exposure in the bottle on the concentration of different VSCs of a

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Sauvignon Blanc wines after 6 months of bottle storage. Thiol compounds such as H_2S, MeSH, and 3MH were affected by oxygen exposure, their final concentration being consistently higher in wines receiving lower oxygen exposure during bottle storage. Conversely, DMS was not affected by oxygen exposure. It is important to notice that the different degrees of oxygen exposure applied in this study were obtained by altering the oxygen content in the closure and/or in the storage atmosphere, while the same closure was always used, which avoided any differences due to possible adsorption of the analytes on the surface of different closures. The higher concentration of thiol compounds observed in wines receiving oxygen exposures in the range of 1.4-3.1mg/L is consistent with the observation that, under conditions of low oxygen availability, these compounds are better preserved in wine, due to lower formation of reactive species that are able to oxidize thiol groups, for example quinones (*21*). It is worth observing that, while the thiol compounds measured in this study all showed the same type of response to oxygen exposure, their sensory role is very different. Indeed, while H_2S and MeSH are mostly associated with unpleasant aroma attributes such as rotten egg, cabbage and sewage, 3MH is considered a key contributor to wine fruity aromas. The achievement of a suitable balance between these compounds having similar chemical reactivity in the wine environment represent a major challenge in the wine industry, especially in the case of light-style fruity driven wines such as Sauvignon Blanc (*22*). The range of oxygen exposure used in this study allowed modulation of different thiol compounds over a relatively broad range of concentrations (Figure 3), indicating that management of oxygen exposure has the potential to assist winemakers in delivering wines with optimal sensory profiles. However, it can be also observed that factors related to wine composition, for example GSH concentration at bottling, act in synergy with oxygen exposure in determining the final concentrations of different thiol compounds. Further research is needed in order to generate adequate knowledge of the effect of different degree of oxygen exposure on wine aroma composition. In any case, it can be concluded that the degree of oxygen exposures and the GSH concentrations applied in this study did not affect DMS accumulation.

Conclusion

Winemaking is a complex process in which different quality control strategies are applied at different steps, with the ultimate goal of improving the final quality of the product. Fermentation practices, including management of juice and must nitrogen content, are mainly considered from the point of view of optimization of fermentation kinetics and yeast-derived aroma metabolites. However, in some cases, these strategies result in outcomes that will emerge later in a wine's life. This appears to be the case for DMS, which was found to form in higher concentration during aging of wines obtained from high DAP-supplemented fermentation. Likewise, the amount of GSH present in the wine at bottling, which is largely dependent on pre-fermentation and fermentation variables, can influence the accumulation of reductive compounds, such as H_2S, during wine bottle ageing. On the other hand, control of oxygen exposure can influence the evolution of different VSCs during bottle aging, which is in line with the common idea of this topic as being typically related to the chemistry of wine bottle aging. Nevertheless, the outcomes of oxygen exposure on VSCs depend on wine composition, and therefore are ultimately linked to the history of the wine in its entirety.

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Chapter 13

The Effect of Pellet Processing and Exposure Time on Dry Hop Aroma Extraction

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The rate of hop aroma compounds extracted from Cascade hops during dry hopping was studied using a model beer system devoid of malt, yeast aromas, and hops. Cascade hops pelletized by four different processors yielded different particle size distributions and pellet densities. These pellets were dosed into a degassed medium (water, 6% v/v ethanol, pH 4.2) and the hop aroma extraction was measured periodically over a one week period. Solid phase micro-extraction (SPME) followed by gas chromatography (GC-FID) was used to analyze the levels of aroma compounds in the extraction medium. Variation in the hop pellet physical properties did not significantly impact the extraction rate of hop volatiles such as linalool, geraniol, limonene and myrcene with one exception. One treatment showed an increased absolute concentration of geraniol. Separately, dry hop aroma extraction was measured over a short time (1 day) at room temperature in an unhopped beer. Irrespective of the hop form (whole or pellet), the concentrations of hydrocarbon terpenes peaked between 3 and 6 hours and subsequently declined, while the concentrations of terpene alcohols continued to increase throughout the 24 hour dry hop extraction. The rate of hop aroma extraction does not appear to be significantly influenced by hop pellet properties and occurs rather rapidly regardless of the hop form.

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Introduction

Hops are used worldwide as a preservative, flavor agent, and aroma source in the manufacture of beer. Hops contain many aroma active compounds, most of which originate in the hop's essential oil. The essential oil is primarily composed of hydrocarbon terpenes such as myrcene and humulene, but also possesses a much smaller oxygenated portion of terpenoids such as linalool and geraniol which are potent odorants in beer (*5*, *6*).

During the beer manufacturing process hops are traditionally added prior to fermentation during a vigorous boil; however, they can be added post fermentation to immature beer in a process known as dry hopping. Because of volatilization during boiling, thermal degradation, and biological transformation via yeast (*11*), hop aromas present in finished beer that has been traditionally hopped often do not resemble the aroma profile of the original whole hop cone. These transformations do not occur appreciably during dry hopping. The thermodynamics of dry hopping are very different from traditional hopping in that dry hopping is usually carried out at 1 to 6°C and there is often little or no agitation of the beer. Thus there is little stripping effects and the oils coming from the hops are retained to a large degree in the finished beer. Because of its volatility, the hydrocarbon fraction of hop essential oil is not typically found in beer that has been hopped using traditional techniques of adding hops to the boil, yet it can be found in appreciable amounts in finished beer when it has been dry hopped. In fact, the oils added during the dry hop regime will closely resemble the oil profile of the raw hops (or hop products).

Dry hopping results in beers with intense hoppy aroma profiles. Traditional hopping followed by dry hopping produces beers that contain both the thermal degradation products of the essential oil that survived the boiling process and yeast-transformed hop compounds as well as the unaltered essential oils coming directly from hops added during the dry hopping process.

The hops used by brewers for dry hopping generally fall into two categories: whole hops or pelletized hops. The former category refers to whole, intact hop cones that have been dried and baled without any further processing. The latter category involves taking whole cones, milling them in a hammer mill to produce a pulverized/powdered hop grist and then extruding the powder through a pelleting die to produce a compact pellet. This results in a hop product that has a much higher bulk density than the former whole cone and a powderized grist that disperses easily upon addition to hot wort. Dispersability in cold, unagitated beer can be affected by the pellet properties, particularly the pellet density. Most of the previous work published on the effect of the pelletizing process on hops has focused on the conservation of α-acids (*2*). With a commercial interest in dry hopping, retention of hop aroma compounds during processing is gaining interest by brewers and hop processors.

Pellet density is partially a function of the die size and speed of extrusion during the pelleting process, which also correlates to heat produced during pellet formation (*2*). All else being equal, less dense pellets should experience less heat during formation, which could result in conserved essential oils and fewer oxidation products. It is recognized as good manufacturing practice to maintain the pelleting temperature between 38°C (100°F) and 50°C (125°F). Operating in this range ensures that the lupulin glands remain liquid but inordinate losses of α-acids and essential oils do not occur (*10*). In other manufacturing processes employing a pelleting process (such as pharmaceuticals), the density of the pellet affects its speed of dissolution. It can then be assumed that hop pellet density affects the speed at which the pellet hydrates and disintegrates in a liquid medium.

The studies presented herein examine how hop oil extraction during dry hopping can be affected by physical properties of the hop material. The first part of this investigation was designed to test the impact of the pellet characteristics on aroma compound extraction rate. Particle size distribution and pellet density were identified as the dominant characteristics that could impact the rate of extraction. Particle size distribution of the hop material varies greatly among pellet manufacturers and is largely determined by the milling process. Smaller particles present more surface area per unit volume of hops potentially resulting in a greater degree of solvent interaction.

The second part of this study was designed to examine the extraction rate of aroma compounds during the initial 24 hour period of dry hopping. Most commercial dry hopping regimes last anywhere from 3 days to 1 week with some brewers dry hopping for up to one month, but it was unknown whether that timeline represents the optimal extraction time for hop aroma compounds or whether it is simply a brewing tradition.

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Materials and Methods

Dry Hop Materials

The week-long extraction study utilized pelletized Cascade hops harvested in 2009 and whole hops harvested in 2010. Three separate lots of pelletized hops each from four different manufactures were obtained and stored at -23 °C until used. The short term extractions utilized Cascade whole hops and pellets harvested in 2010 from the same hop farm.

Dry hopping was carried out in a model beer system consisting of acidified, filtered water (94%) and ethanol (6%). The solution was buffered at pH 4.2 with sodium citrate/citric acid (0.0116 M). The water was degassed by boiling and then cooled prior to blending with ethanol and acid. The solution was dispersed in 18 L aliquots into modified Cornelius kegs and cooled to 1 °C prior to dry hop dosing.

The short term aroma extraction study was conducted using smaller scale bench top equipment. Each sample was extracted in a 0.5 liter sealed, brown glass bottle that had been flushed with nitrogen. The extractions were performed using both the model beer solvent and unhopped beer brewed specifically for this study. The unhopped beer was brewed using 98% pale 2-row malt and 2% acidulated malt. Alpha acids (from CO_2 extract) were added at the beginning of a 60 minute boil at a concentration of 12 ppm. Original gravity was 1.0442 (11° Plato) and final apparent gravity was 1.0047 (1.03 ° Plato) after fermentation with an ale yeast at 18 °C.

Standard curves of hop aroma compounds were prepared using analytical grade chemicals (Sigma-Aldrich Corp, St. Louis, MO). Direct oil injections were dissolved in hexane, which was redistilled prior to use.

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Dry Hop Method

The week-long dry hopping experiments were carried out by adding 23.2 grams (1/3rd pound/barrel or 127 g/hL) of hop pellets to a chilled model solution in a sealed stainless steel keg that was flushed with CO_2. An equal mass of whole hops was placed into a mesh bag and kept submerged about 6 cm from the bottom via an inert stainless steel weight. Following the addition of the hops, the keg's headspace was flushed with CO_2 three times to ensure little to no oxygen remained, and the headspace pressure was reduced to ambient pressure. There was no agitation of the systems during the dry hopping trial. Samples (20 mL) were removed via a shortened dip tube after 1 day, 4 days, and 7 days. The shortened dip tube reached to the middle of the keg and allowed a drawn sample that contained no visible vegetative hop matter. Each of the 16 hop treatments was used once during this study. Thus, the replication of the hop treatment was dealt with by using 3 independent Cascade hop samples from each of the 4 suppliers, plus one single, whole hop sample.

For the short term extractions, dry hopping was also performed at a dose of 1/3rd lb. per barrel (127g/hL). The extractions were performed at room temperature (20°C). After hop dosing, the headspace of each bottle was flushed with nitrogen to limit oxidation and then sealed. The bottles were agitated using a shaker table so that diffusion from the hop particles to the medium would be maximized.

Extractions were sampled at 30 minutes after dosing and at various intervals over 24 hours. After sampling, the extraction bottle's contents were discarded, thus each sampling point can be considered an individual treatment.

Pellet Characteristics

Pellet density was measured using a bench top micrometer (Mitutoyo Corp, Model: SDV-6"A,) and an analytical balance (Sartorius, Model: R16OD, Goettingen, Germany). Each measurement included 10 randomly chosen pellets. Hop pellets were treated as a cylinder for purposes of calculating volume. Where needed, the ends of the pellets were straightened with a razor to create uniform cylinders.

Particle size was measured using a five sieve system utilizing U.S. standard sieve sizes: 2.36mm, 1.20mm, 0.59mm, 0.25mm, and 0.15mm (Dual Manufacturing, Chicago, IL). Samples were prepared by first dispersing pelletized hops in 20°C water then drying the particulate matter overnight on a screen. This method was preferable to disintegrating the pellets manually or via crushing under a rolling pin as it prevented any further milling effect from occurring during sample preparation. The dried sample was then placed in the sieve system and shaken via a mechanical shaker for five minutes. Retained portions from each sieve were weighed and recorded. Percent retained (as a percent of total mass) was calculated, as well as an aggregate weighted mean diameter. The weighted mean diameter was calculated as per the ASBC standard method for malt grist analysis (*1*).

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Solid Phase Micro-Extraction

Hop oils transferred to beer or model beer solution via dry hopping were measured using a headspace solid phase micro-extraction (SPME) technique. A 10 mL of sample was loaded into a 40 mL amber glass vial with a Teflon-lined silicon septum which was placed in a 45 °C circulating water bath. A 2 cm tri-phase fiber, consisting of polydimethylsiloxane, carboxen, and divinylbenzene (PDMS/CB/DVB) with a 50/30 μm coating thickness was inserted in the headspace above the solution in the glass vial and volatiles were allowed to adsorb to the fibers during a 60 minute extraction period. During the extraction, the sample was stirred by a glass-coated magnetic stir bar at 500 RPM. 4-octanol was added as an internal standard during SPME sample preparation at a final concentration of 1 ppm for longterm extractions and 0.5 ppm for short term extractions.

Short term extraction samples were also dosed with 2g NaCl. Because of the nature of the extraction (shaker table agitation), the short term extraction samples included an additional filtration step using a 0.45 micron cellulose syringe filter. Samples were prepared and analyzed within one hour of being drawn.

Gas Chromatography

Volatiles adsorbed to the SPME fiber were identified and quanitified using gas chromatography (GC) analysis via a Hewlett Packard 5890 with a flame ionization detector (FID). Detector temperature was 250 °C. The column was a Supelcowax 10, 30m x 0.25mm x 0.5μm (Supelco, Bellfonte, PA). Carrier gas was nitrogen with a flow rate of 1 mL/minute (splitless mode for SPME, 1:50 split ratio for oil direct injections). Desorption of volatiles from the SPME fiber was performed at 250°C for 10 minutes. Oven temperature started at 50°C, and underwent the following temperature ramp: 50°C for 1 minute then at 4°C/min to 90°C, 5°C/min until 185°C, hold for 6.5 minutes, 3°C/min until 230°C and hold for 10 minutes. SPME injections and oil direct injections utilized the same temperature program, but all SPME injections were conducted manually whereas direct injections of oil samples were performed using an auto sampler to minimize injection volume variation. The oil analysis followed the standard ASBC method (*1*).

Essential oil content of each pellet type was measured via steam distillation, which was carried out according to the ASBC standard method (*1*). Distilled oil volume was recorded and a portion was retained and stored at 4.5°C for further analysis.

Results

Pellet Density

Pellet process treatments had a significant effect on pellet density (Figure 1). Group 1 (Pellet C and Pellet A) were not significantly different from each other, likewise group 2 (Pellets A, B, C) were not significantly different from one another (Tukey's HSD test, α=0.05).

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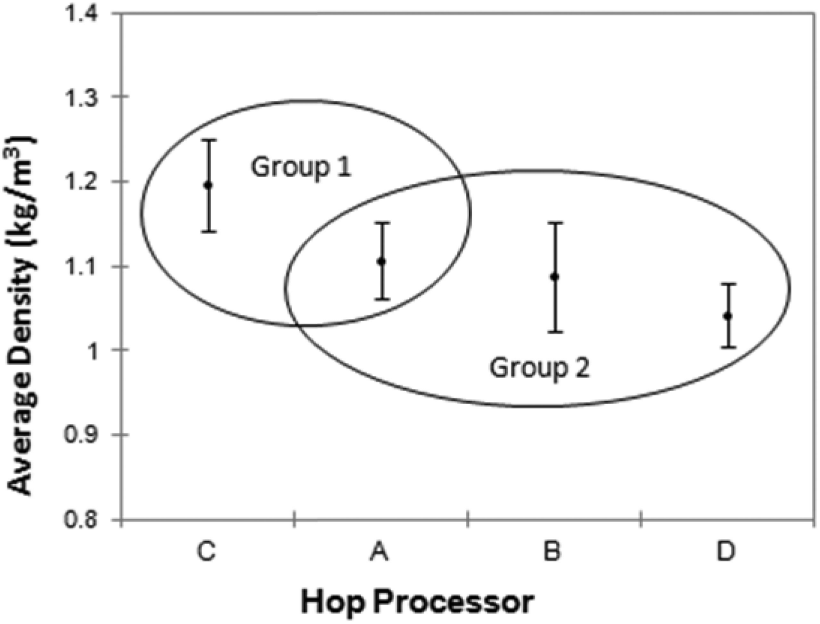

Figure 1. Hop Processor's Pellet Density. N=3, mean values ± one standard deviation. Means within the same group are not significantly different at α = 0.05.

Pellet Particle Size

The hop grist particle size varied significantly from producer to producer. Analysis of variance of the hop pellet particle size data showed that Pellet D's particle size distribution was significantly larger than distributions from Pellet C (P=0.031), Pellet A (P=0.013), and Pellet B (P=0.0025). Pellet C was significantly larger than Pellet B (P=0.0037). Pellet A did not significantly differ from Pellet B or C.

The aggregate weighted mean diameters for each pellet type are shown in Table 1.

There was a lot of unsorted information above the largest bin (2.36mm) that remained unresolved for the two treatments with the largest particle sizes (Pellets D and C), so their aggregate mean particle diameter could potentially be slightly higher.

Table 1. Aggregate Weighted Mean Diameter

Hop Processor	*Mean Diameter*
Pellet D	1.72 mm
Pellet C	1.37 mm
Pellet A	1.09 mm
Pellet B	0.95 mm

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Long Term Dry Hop Aroma Extraction

GC chromatograms were obtained for each sample (3 per treatment, 3 timepoints). Figure 2 shows the average concentration of linalool at days 1, 4, and 7. Figure 3 shows those same time points for the compounds myrcene. Surprisingly, extraction data did not show an increase in compound concentration over the time periods examined; in all cases the day 7 concentrations were either near the same level as day one (within standard deviation) or had fallen slightly. Final concentrations did not significantly differ between treatments, with the exception of geraniol.

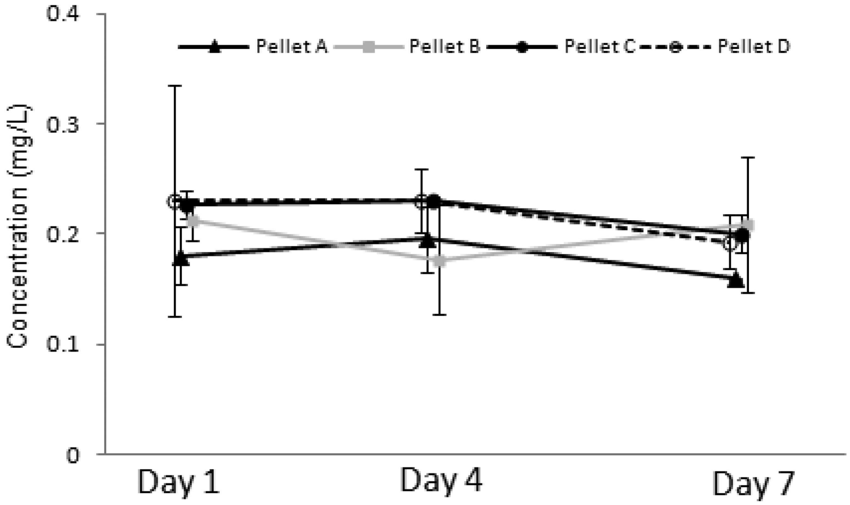

Figure 2. Average linalool concentration at Days 1, 4, and 7.

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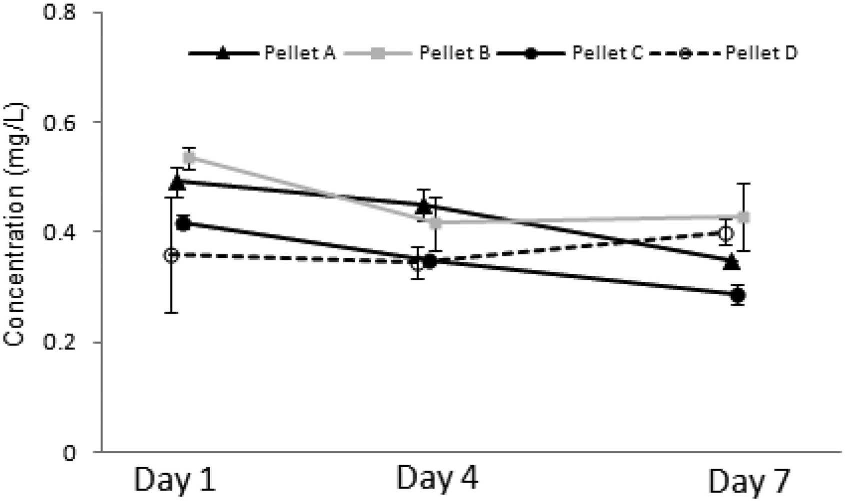

Figure 3. Average myrcene concentration at Days 1, 4, and 7.

Short Term Dry Hop Aroma Extraction

The results from GC analysis of the short term, agitated aroma extraction showed that hydrocarbon compounds are fully extracted in as little as 4 hours. The overall trend for hydrocarbon compounds is a rapid increase in concentration followed by a decline during which the rate of decline flattens out. In contrast, the terpene alcohols appear to extract rapidly at first and then either remain static, or increase very slowly over the extraction period. Figures 4-5 show the concentrations for aroma compounds from 30 minutes out to 24 hours.

Discussion

Week-Long Extractions

Early, bench-top observations of pellet dispersals revealed that in all cases pellets disintegrated in cold water in less than thirty minutes; on a dry hop timescale of 24 hours to one week the dissolution time would be irrelevant. Thus, the differences in pellet density did not affect disintegration rates. The pattern of dispersal, however, varied greatly among pellet types with some pellets dispersing and then coalescing on the bottom of the vessel and others forming one layer near the surface of the medium and another on the bottom of the tank. This behavior is assumed to be related to pellet density and particle size. While this pattern of dispersal may affect extraction in the short term, no effect was seen during the longer intervals tested in this work.

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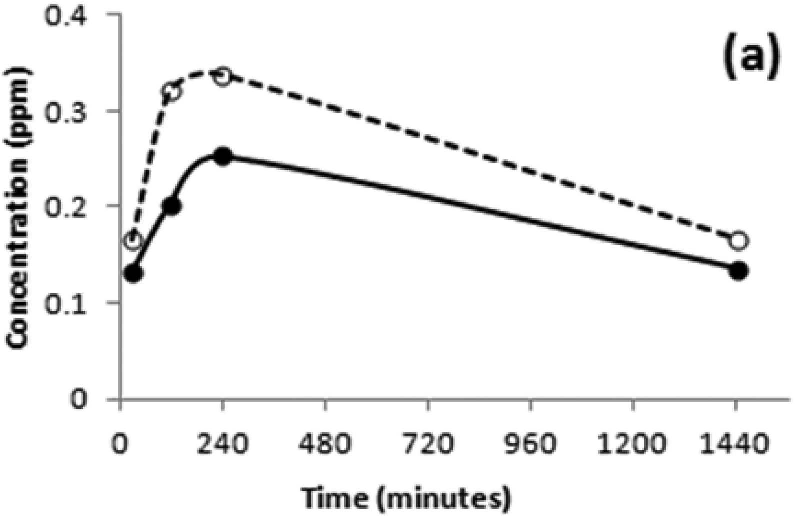

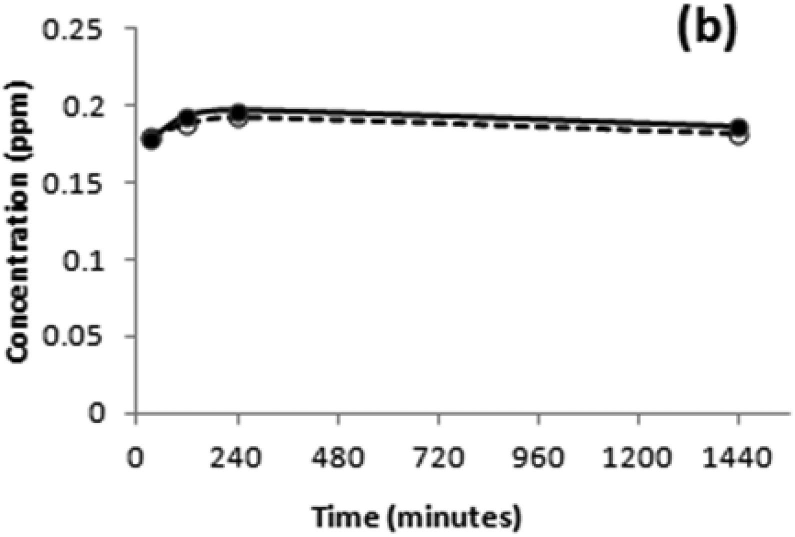

Figure 4. Myrcene (a) and humulene (b) concentrations during a 24 hour dry hop treatment with pellets (○) or whole cone hops (●).

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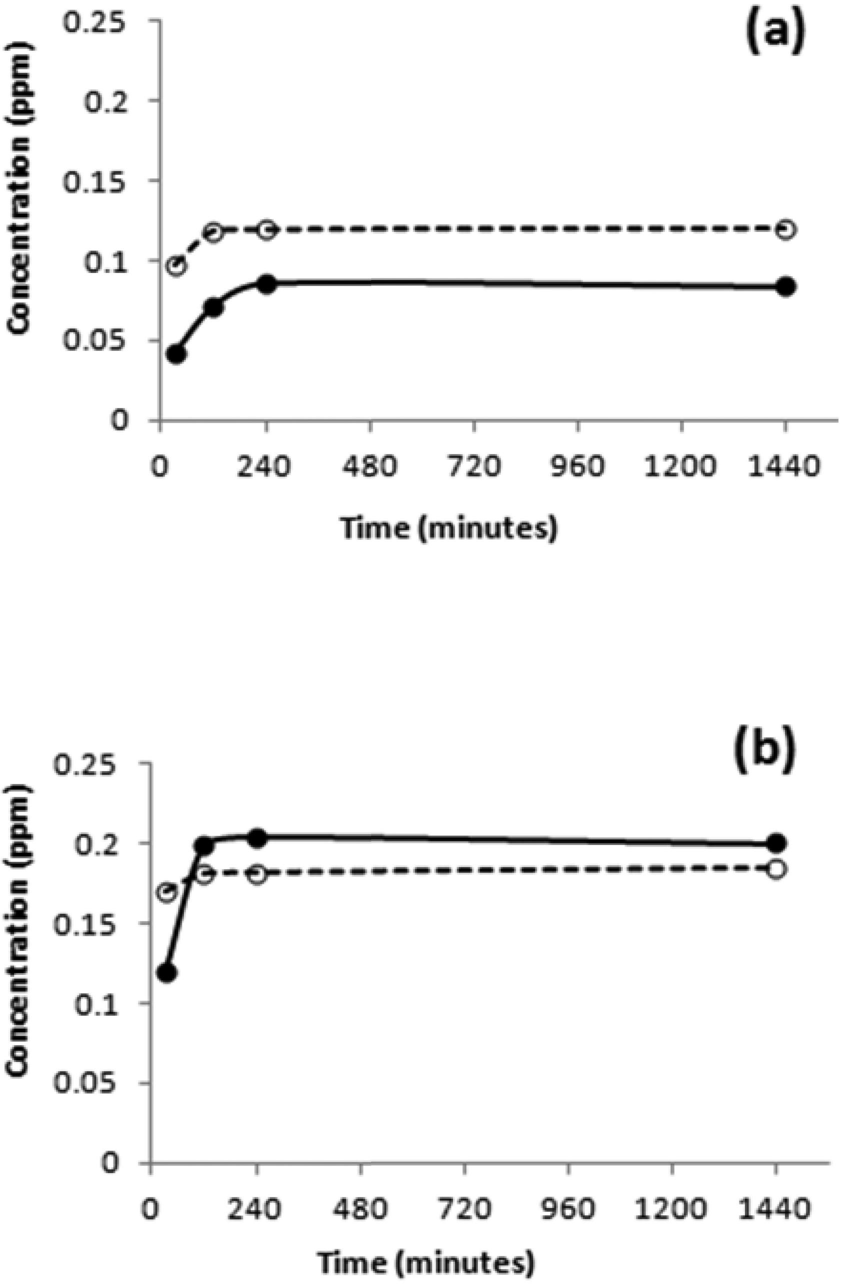

Figure 5. Linalool (a) and geraniol (b) concentrations during a 24 hour dry hop treatment with pellets (○) or whole cone hops (●).

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The pellet particle size data also reflected what a hand inspection revealed; Pellet B pellets were powdery when broken apart, whereas the Pellet D pellets most closely resembled ground whole hops and had recognizable hop cone bracteoles. There was a loose correlation (data not shown) between particle size and the tendency for particles to stay in suspension near or on the surface or settle out on the bottom of the tank, with the smallest particles tending to settle out. While this behavior is interesting and may have some brewing process ramifications during tank cleanout or whirlpooling, no treatment effect was seen on aroma compound extraction rate in the present study. This is likely because extraction occurred outside of the timeframe we observed in the week-long extraction study.

Each of the four suppliers produced pellets with different densities which were apparent to the eye. The pellet density mirrored the physical inspection of the pellets with the densest pellets possessing a reflective sheen associated with exposure to excessive heat during processing (*10*).

Headspace sampling of hop aroma volatiles via solid phase micro-extraction was selected for this work because of its relative simplicity and reproducibility when dealing with hydrophobic, volatile analytes. It allowed immediate analysis of samples taken directly from the dry hop tanks with no further modification, and has been previously used in similar systems with great success (*4*, *9*). While SPME proved to be effective here, other methods of analysis (such as stir bar sorptive extraction) should not be overlooked and could easily be adapted to the same system.

Typical extraction curves in food applications (such as aqueous extraction of tea leaves) have a positive slope indicating an increase in compound concentration over time with an exponential rise to an equilibrium concentration. It was expected that the dry hop extraction data would follow this pattern. The fact that these data instead showed no positive trend with time indicates that the extraction may have been complete by the time the first samples were analyzed.

Analysis of variance showed that there were significant differences in the physical properties among the pellet treatments examined. However, these differences did not significantly affect the extraction rate of the terpene and terpenoid compounds between day one and day seven. These data indicate that the extraction of aroma compounds may occur much faster than the typical commercial dry hopping regime of several days to several weeks; terpenes may reach their solubility threshold in a matter of hours instead of days. These data were the impetus for the short term extraction experiments.

While our study was designed to examine rate of extraction, the final concentrations themselves deserve attention. The final concentrations of linalool, myrcene, and limonene were not grossly different among treatments with one exception. Pellet D showed a treatment effect with respect to geraniol concentrations (data not shown); the final geraniol concentration from Pellet D was significantly higher ($p<0.001$) than the other treatments. Geraniol contributes a floral and ester note to the aroma of beer (*8*).

The oils present in the hop pellets was examined first distilling the oils from the pellets in an aqueous boil using standard methods (*1*) followed by chromatographic separation and analysis. The hop oil analysis showed that the pelleting process tended to reduce overall myrcene levels and increase levels of oxidation products. This agrees with a large body of previous work (*2*, *3*, *10*). In particular, Pellet C samples showed high levels of oxidation products (humulene oxide and caryophyllene oxide). Pellet C samples also had the greatest density, and although this study did not attempt to correlate these data, it is possible the more intense pelleting process (as inferred by the highest density) had a direct effect on oxidation levels of the oils in these pellets.

When looking at the oil data across all treatments, there was sufficient variability in the replicates within each processor that there appeared to be little difference among the pellet treatments beyond the oxidation products for the Pellet C samples. The Pellet C samples had greater variation than the other three producers. While the single sample of whole hops had no measure of sample variation, it was highest in myrcene and very low in humulene epoxide and caryophylene oxide (oxidation markers).

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Short Term Extractions

As expected based on the data from the long term extraction, the extraction of hop aroma compounds occured much faster than the interval of days or weeks typically used in commercial breweries. These data displayed peak concentrations typically occurring around 300 minutes. Bearing in mind that these extractions occurred at 23°C and were continually stirred, this is still much faster than we initially expected. If the extractions occurred at the more typical temperature of 1-4°C, peak concentration would take longer to achieve but would still probably occur in under 3 days. Note that the work by reserachers at the Technical University of Munich in Weihenstephan (discussed below) had hop aroma peak intensity during bench top dry hopping experiments occurring at approximately 3 days during a stirred dry hop extraction at 1°C .

Following their peak concentrations, the terpene alcohols (linalool and geraniol) and hydrocarbons (myrcene, humulene, and limonene) exhibited dichotomous behavior. The terpene hydrocarbons were unstable in both the beer matrix and the model system and declined in concentration (Figure 4). The terpene alcohols were stable and either maintained their peak concentrations in the beer matrix (Figure 5) or continued to slowly increase in the model system (data not shown). Similar results were found by Krottenthaler et al. (*7*). They observed no change in linalool and geraniol concentration over a 1 week extraction. Their hydrocarbon data was slightly different with a longer time required to rise to maximum at day 3 and then a subsequent decline; this time difference can be explained by a lower extraction temperature (0°C) as compared to that used in the studies presented herein (23°C). While they found a dose-response effect, they were equally surprised to see no change in polar compound concentrations with time.

Regarding the form of the hop material, pellet dosing resulted in a larger concentration of extracted compounds relative to whole cone hops for all samples taken at the initial 30 minute time point. There are a couple of hop pellet characteristics that may account for this. Firstly, the hop material in pellets has a greater overall surface area relative to whole cones because of their smaller milled particles. Secondly, the lupulin glands, which contain hop essential oils, have been crushed and distributed throughout the vegetative matter during the milling and pelleting processes. Both of these factors expose more essential oil for extraction immediately upon the pellet's dissolution. However, this initial jump in concentration did not always result in a higher concentration after 24 hours of extraction.

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Chapter 14

HPLC-ESI-MS Identification of Hop-Derived Polyphenols That Contribute Antioxidant Capacity and Flavor Potential to Beer

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Beer is one of the most commonly consumed beverages world-wide and it is nearly always brewed with hops (*Humulus lupulus, L.*). Although hops contribute a mere fraction of the beer raw ingredient bill, the use of hops immensely impacts the flavor and quality of finished beer. Hops can provide beer with bitterness, aroma, flavor and texture and also enhance specific beer properties such as foam stability, clarity (colloidal stability), color, flavor stability and microbial stability. Beer prenylflavonoids represent a class of antioxidant compounds that are generally referred to as polyphenols. In this study a polyphenol rich extract was prepared from spent hop solids. Dosing this extract into a commercial lager beer indicated that hop polyphenols provide anti-staling capacity (antioxidant capacity) and may also affect beer flavor profiles (sensory analysis). Fractionation of the extract allowed for identification of several classes of prenylflavonoids that could be further correlated with varying levels of antioxidant character via DPPH• antiradical capacity.

In Flavor Chemistry of Wine and Other Alcoholic Beverages; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Introduction and Justification

Hops (*Humulus Lupulus* L., Cannabinaceae) are an essential raw ingredient used in the brewing industry to bitter beer. Hops also provide beer with other quality attributes such as microbial stability, foam stability, mouthfeel, color, flavor and flavor stability (*1–7*). Some of these parameters are thought to depend heavily on the hop polyphenol content of finished beer. As a result, hop polyphenols have become the focus of many investigations focusing on beer stability. However, results of these studies diverge: some indicate that polyphenols of the flavonoid family have a protective effect on beer flavor stability (*8–12*), while others imply that polyphenols either have no impact or negatively impact beer flavor stability (*13–17*).

A number of hop products are now commercially available for use throughout the brewing process: pelletized hops, pre-isomerized pelletized hops, resin concentrated pelletized hops and resinous extracts (prepared by critical or liquid CO_2 extraction) (*4*, *6*, *7*, *18*). Consequently, the use of hop pellets has been in decline, leaving a large portion of the hop cone as a waste stream of spent hop solids/powder. This spent hop solid material is generally rich in polyphenols (*5*, *19*).

The variety of polyphenols found in hop plant materials complicates the identification of individual compounds, and hop solids – although having already been extracted and stripped of bittering resins by soft critical or super-critical CO_2 extraction- are no exception. Comparison of retention times and U.V. spectra of compounds in question to a known reference does not always provide adequate information to allow for unambiguous identification of individual compounds. However, HPLC-UV-DAD and MS/MS can assist in the partial structural elucidation and identification of polyphenols.

Research conducted in this study suggests that hop polyphenols have something of interest to offer the brewer in terms of flavor and flavor stability (*5*, *7*, *19*). A polyphenol rich extract prepared from spent hop solid materials (*Humulus lupulus* L. cv Galena) was produced using Amberlite FPX adsorption resin. The extract was dosed at a rate of 100 ppm total polyphenols into lager beer and the beer was aged for eight weeks under cold and accelerated storage. The added polyphenols were found to improve the antioxidant capacity of a commercial lager beer as measured by antioxidant (FRAP and DPPH•) and anti-radical (ESR) assays (*8*) and sensory analysis revealed that hop polyphenols also contribute to beer flavor. Reverse-phase (C18)- HPLC-ESI-MS chromatography in conjunction with phloroglucinolysis revealed that the extract was nearly 99% phenolic in nature, with low levels of proanthocyanidins (2% by mass), traces of procyanidin monomers, B-type dimers and a plethora of other compounds that are suspected to be xanthohumols, flavonols, flavanonols and their glycosylated counterparts. However it was unclear as to which of these compounds, if any alone or in synergy, were responsible for the improved anti-oxidative/anti-radical response elicited by the dosed extract.

Assessing the anti-oxidative effect(s) of an individual compound or class of compounds on food systems or in living systems can be a complicated affair. Not all systems are alike and limitations of solubility and bioavailability further complicate matters. In this study the goal was to assess the potential for hop derived compounds to affect beer flavor stability. Therefore, our plan of attack involved combining several methodologies in hopes of determining which compounds found in the polyphenolic extract were responsible for improving lager beer flavor stability.

Materials and Methods

Preparation of Hop Solids

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Spent hop-derived polyphenol rich extracts were produced at lab scale: 450 mL of a spent hop solid (*Humulus lupulus* L. cv Galena) aqueous extract was prepared by extracting 45.25 g of spent hop material in 1 L of water under simulated kettle boiling conditions (pH 3.0, 3N HCl) . Acidification was done in effort to minimize solubilization of residual alpha acids. The mixture was heated to 40°C and held there for 30 min. Wheat gliadin (suspended in 95% ethanol) was added to the mixture at 0.5% w/w in order to mimic action of wort proteins in the kettle and boiled for 30 min. Following a coarse filtration with cheese cloth, the extract was refrigerated overnight (4°C), centrifuged (16,000 g, 15 min. at 10°C), alkalized (pH 7, 5N NaOH), treated with EDTA (10g/L) to reduce pro-oxidative metals (Cu and Fe), filtered through a Whatman No. 1 and re-acidified (pH 3.0, 3N HCl).

The aqueous extract was applied to a 4.5×15cm Chromaflex (Kontes, Vineland, NJ) preparative column containing a high-capacity Amberlite™ FPX66 food grade adsorbent resin, rinsed with 1.0 L of MQ water, and the polyphenols of interest were eluted with 300 ml of 95% EtOH. The eluted fraction was then diluted with 50 mL of MQ water and further concentrated by roto-evaporation (30°C), and subsequently freeze dried to yield 0.94 g of a light yellow fluffy powder (polyphenol isolate).

Preparation of Hop Solutions

To 5 mL of MQ water, 0.11 g of the polyphenol isolate was added and sonicated until solubilized. The entirety of the 5 mL aqueous solution was applied to a preconditioned (95% ethanol, followed by MQ water) C-18 solid phase extraction cartridge (60 mL, 10 g, Supelco, Bellefonte, PA). The compounds of interest were eluted with 60mL effluent in the order of solvent polarity. Eight fractions were collected: 0%, 10%, 20%, 30%, 40%, 50%, 70% and 100% of 95% ethanol/water. The fractions were concentrated under roto-evaporation (30°C) to a constant volume of 5 mL (hop solutions). The polyphenol isolate was added and extracted using C-18 separately for each fraction, i.e. a 5 mL aqueous solution was prepared using the polyphenol isolate 8 times, applied to a new column each time and eluted with 60 mL of effluent ranging in polarity from 0-100% ethanol (95%) (polyphenol fractions).

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Total Polyphenols and Total Flavanoids

Total polyphenols and total flavanoids were measured according to the EBC Analytica methods (9.10 and 9.12) (*20*) using a Shimadzu PharmaSpec UV-1700 spectrophotometer, Shimadzu Corporation (Columbia, MD).

DPPH• Radical Bleaching Assay

To a 10 mL test tube, 2 mL DPPH• stock reagent (2.9 mg/50 L Methanol) was added. 50 µL of hop solution was added, vortexed for 20 seconds, incubated for 10 minutes at room temperature and the absorbance was read at 518 nm. %DPPH• reduction = [(Absorbance 518 nm DPPH• – Absorbance of the test sample)/ Absorbance DPPH•] x 100%

HPLC/ESI-MS

The reversed-phase method consisted of two Chromolith RP-18e (100- 4.6 mm) columns connected in series with accompanying guard column (Chromolith RP-18e, 5-2.6mm) all purchased from EMD chemicals (Gibbstown, NJ). The procedure utilized a binary gradient of 1%v/v aqueous acetic acid (A) and acetonitrile containing 1%v/v acetic acid (B). Eluting peaks were monitored at 280 nm: 1.0mL/min; 5% B at 0 min, linear gradient from 5- 10% B, 0-10 min; 10-30% B 10-20 min; 30-55% B, 20-40 min.; 55- 90%B 40-41min.; 90%B, 41-51 min. The column was washed with 5% B for 5 minutes prior to the next injection. For the ESI source, the following conditions were applied; negative mode, dry temperature 350°C; dry gas 10.0 L/min; nebulizer 50.0 psi, trap drive 47.5, skim 1 -38.3 volt, skim 2 -6.0 volt, octopole RF amplitude 120.0Vpp, capillary exit -113.0 volt, scan begin 50m/z, scan end 1800 m/z.

Results

Analytical results are depicted in Figure 1. As seen in Figure 1 three fractions, 10%, 20% and 30% exhibited the greatest antiradical effects as assessed by DPPH• radical quenching. Fractions of 10% and 20% ethanol were highest in total polyphenols and flavanoids.

Structural analysis of the polyphenolic components of fractions 2-8 was conducted by RP-C18 HPLC-ESI-MS (negative mode). Fraction 1 was not analyzed via HPLC-ESI-MS. The identification of the polyphenols in each fraction was complicated; hundreds of compounds were detected. Currently, we have proposed the identity of many compounds, however many remain unidentified. Further analysis by MS/MS may assist to elucidate the identity of the compounds we were not able to propose identities for at this time. Structures of some of the known flavonoid polyphenols found in this study are presented in Figure 2.

In Flavor Chemistry of Wine and Other Alcoholic Beverages; Qian, M., et al.;
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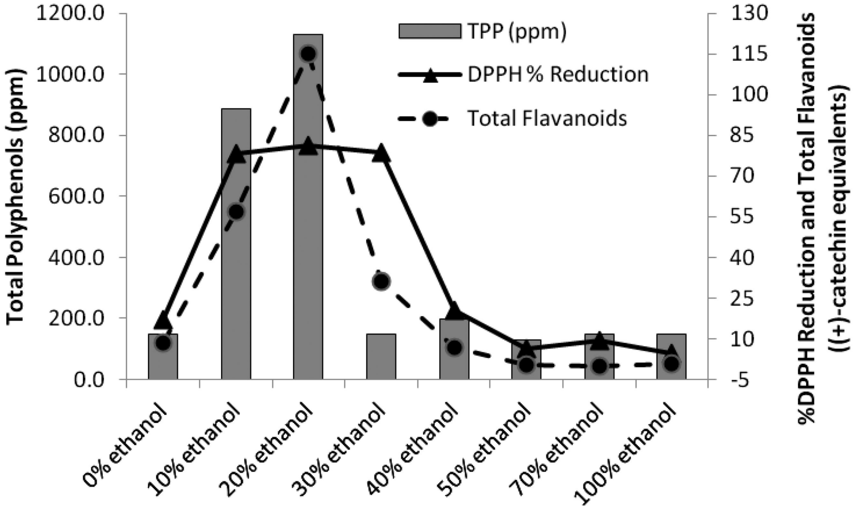

Figure 1. Polyphenol fraction impact on total polyphenols, total flavanoids and DPPH• activity.

Fraction 2 (10% ethanol) was high in total polyphenols and total flavanoids and also contributed the greatest antioxidant potential as measured by the DPPH• radical capacity assay. HPLC-ESI-MS results indicate that most of the components eluted between 0 – 10 minutes (retention time). A wide variety of compounds were found, which are tentatively characterized in Table I. (+)-Catechin dimers and trimers, prodelphinidin dimers, hop bittering related compounds such as desoxy-alpha-acids, lupulone, and tetrahydrolupulone were tentatively identified.

Fraction 3 (20% ethanol) components eluted between 10 and 25 minutes retention time. This fraction was found to contain xanthohumol derivatives and humulinones (oxidized humulones).

Fraction 4 (30% ethanol) was found to contain several glucosides and rutinosides of quercetin and kaempferol which eluted between 15 and 25 minutes retention times. Fraction 4 was found to be low in total polyphenols, total flavanoids as measured by the spectrophotometric methods. However the DPPH• capacity assay indicated that fraction 4 had substantial anti-oxidant capacity, equivalent to fractions 2 and 3.

Fraction 5 (40% ethanol) was lower in total polpyhenols and total flavanoids and also weak in DPPH• antiradical capacity. Four peaks dominated fraction 5 which were characterized as quercetin-3-0-rutinoside, quercetin-3-O-glucoside, kaempferol-3-O-rutinoside, and kaempferol-3-O-glucoside.

Fractions 6, 7 and 8 consisted of low total polyphenols and total flavanoids and also had week DPPH• antiradical capacity. Multiple minor components eluted in these fractions : 25-40 minutes, 15-40 minutes, and 45-55 minutes respectively. These fractions were found to contain dihydrocohumulone, oxidized-alpha-acids (cohumulinone and humulinone), humulone, colupulone, and xanthohumol. Interestingly, many unknowns found in fraction 7 show a pattern of $[M-1]^{-1}$ + CH_3COO-Na (82) adducts: m/z: 427.2, 509.2; 471.3, 553.3; 515.3, 597.3; 603.4, 685.4; 647.4, 729.3; 691.4, and 773.4.

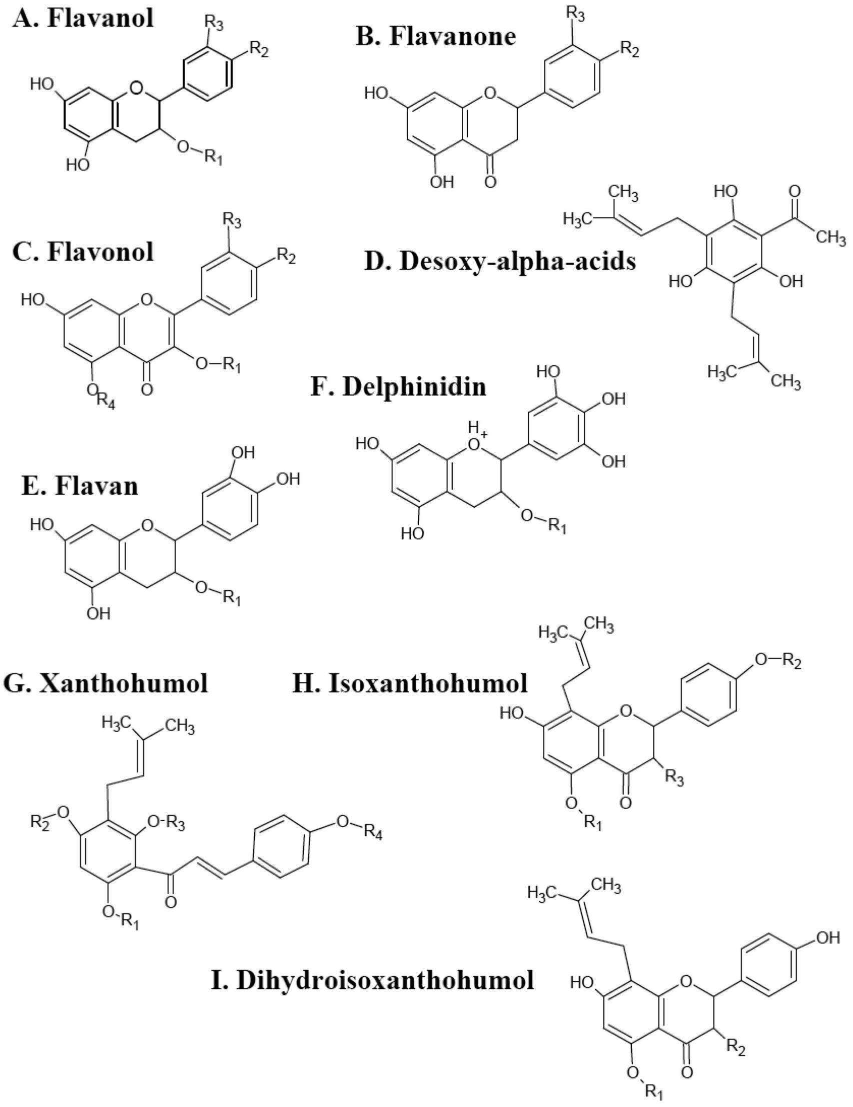
A. Flavanol
B. Flavanone
C. Flavonol
D. Desoxy-alpha-acids
E. Flavan
F. Delphinidin
G. Xanthohumol
H. Isoxanthohumol
I. Dihydroisoxanthohumol
HO
OH
O
O–R1
O–R2
O-R3
O–R4
R1
R2
R3
R4
H+
H3C
CH3

Figure 2. Structures of proposed compounds found in Table I.

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Table I. Proposed Identity of polyphenolic compounds found in hop extracts via HPLC-ESI-MS; letters A, B, C, D, E, F, G, H, and I refer to structures in Figure 2

Name/Formula	*[M-H]⁻*	*Major Ions (m/z)*	*R1*	*R2*	*R3*	*R4*
Fraction 2						
$C_{17}H_{16}O_6$ (A), (B), (C)	314.8	288.8, 271.8, 270.8, 110.8	H	OCH_3	OCH_3	
				OCH_3	OCH_3	
			H	OH	OCH_3	H
Desoxycohumulone (D)	330.9	242.8, 199.7, 167.7				
Desoxyhumulone (D)	344.8					
$C_{21}H_{14}O_9$ (E)	410.9	345.9, 344.8, 304.6, 290.7, 289.7, 288.7, 272.6, 260.7, 240.7, 180.8, 174.5, 164.7, 149.8	$C_6H_5O_2$ (dihydroxybenzene)			
Unknown	765.1	763.1, 737.1				
Unknown	564.9	476.7, 283.8, 282.9, 281.9, 149.8				
Unknown	345.9	327.9, 311.8, 210.7, 133.8				
Unknown	508.9	486.8, 294.9, 292.8, 259.7, 243.8, 242.8199.8, 109.9				
Unknown	827.1	664.9, 484.9, 325.0, 182.7				
Unknown	324.9	254.8, 211.8, 210.8, 166.8				

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Table I. (Continued). Proposed Identity of polyphenolic compounds found in hop extracts via HPLC-ESI-MS; letters A, B, C, D, E, F, G, H, and I refer to structures in Figure 2

Name/Formula	***[M-H]$^-$***	***Major Ions (m/z)***	***R1***	***R2***	***R3***	***R4***
Fraction 2						
Unknown	327.9	269.9, 210.7, 133.8				
Unknown	422.9	345.9, 260.7, 210.7				
Unknown	647.0	370.9, 359.9, 326.9, 139.8				
Unknown	462.9	328.9, 213.8, 141.8				
Lupulone	412.9	338.0, 290.0, 280.9, 254.7, 161.8				
Unknown	395.0	380.0, 360.9, 179.8				
Unknown	430.9	395.9, 395.0, 371.9, 370.9, 208.7, 136.8				
Unknown	613.0	546.9, 413.0, 382.9, 381.9, 352.9, 205.8, 190.7, 115.9				
$C_{21}H_{20}O_{11}$ (C)	446.9	395.0, 323.0, 194.7, 151.8	glucoside	H		H
Unknown	352.9	190.7, 178.7, 134.7				
Catechin dimer (2E)	577.0	443.0, 336.9, 288.8, 190.8, 162.8	H			
tetrahydrolupulone	416.9	356.9, 354.8, 336.8, 322.9, 194.8, 162.8				
Unknown	336.9	321.0, 222.7, 208.8				

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Name/Formula	***[M-H]⁻***	***Major Ions (m/z)***	***R1***	***R2***	***R3***	***R4***
Fraction 2						
Catechin trimer (3E)	865.0	426.9, 409.1, 368.9, 288.8, 222.7, 204.7, 192.8				
Unknown	366.9	352.9, 192.8, 133.8				
Delphinidin dimer (F)	604.1	378.9, 192.8	delphinidin			
Unknown	409.2	351.2				
Unknown	379.2	367.0, 283.1				
Fraction 3						
Unknown	443.2	297.2, 245.1				
Methylated Xanthohumol (G)	395.0	360.9, 350.8, 313.1, 296.8	CH3	CH3	CH3	CH3
Unknown	427.0	374.9, 352.9, 178.7				
$C_{22}H_{18}O_8$ (E)	409.0	395.0, 350.9, 336.9, 284.9	$C_7H_5O_2$ hydroxybenzoate			
Unknown	425.0	378.9, 354.8, 307.8, 208.7, 190.7, 162.7				
Isoxanthohumol+ Dihydroisoxanthohumol (H+I)	707.0	645.9, 353.9, 352.9, 291.9, 190.7	CH3	H	I	
Xanthohumol (G)	352.9	266.8, 192.8, 178.8, 172.8, 134.8	CH3	H	H	H
Unknown	595.0	430.9, 400.9, 361.0, 268.9, 253.0, 192.8				

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Table I. (Continued). Proposed Identity of polyphenolic compounds found in hop extracts via HPLC-ESI-MS; letters A, B, C, D, E, F, G, H, and I refer to structures in Figure 2

Fraction 3						
Dihydroisoxanthohumol (I)	518.9	393.0, 356.9, 335.1, 307.9, 194.7, 192.7	glucoside			
Unknown	379.0	336.9, 288.8, 172.7				
Unknown	379.0	370.9, 300.9, 192.7, 176.8				
Unknown	395.2	381.2, 377.2, 361.2, 333.2, 311.1				
Unknown	578.9	393.0, 244.8, 202.7				
Unknown	693.0	670.9, 356.9, 290.9, 248.9, 194.8				
Unknown	381.0	363.0, 334.5, 280.8, 279.1, 262.7, 190.8				
Unknown	693.0	393.0, 357.9, 356.9, 290.9, 194.8				

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Fraction 3						
Dihydrohumulone /cohumulinone	363.0	331.9				
Humulinone	377.0	364.1, 362.9, 236.8, 190.7				
Fraction 4						
Quercetin-5-O-glucoside-3-O-rutinoside (C)	771.3	427.2, 300.0, 299.0191.0	rutinoside	OH	OH	gluco-side
Kaempferol-5-O-glucoside-3-O-rutinoside (C)	755.1	625.2, 463.1	rutinoside	OH	H	gluco-side
Flavanol (E)	611.3	479.1, 534.2, 480.1, 479.1431.1, 316.0, 166.9	rutinoside			
Unknown	739.1	667.2, 609.0, 394.0, 393.0, 334.0, 333.0, 263.9				
Quercetin-3-O-rutinoside (C)	609.0	597.0, 394.0, 333.0, 289.1, 254.0	rutinoside	OH	OH	H
Qucertin-3-O-rutinoside (C)	463.1	300.0	glucoside	OH	OH	H
Unknown	597.2	463.1, 300.0				
Unknown	715.0	693.0, 405.0, 357.9, 356.9, 194.8				
Kaempferol-3-O-(6”-O-malonylglucoside) (C)	533.0	464.8, 463.9, 462.9, 299.8	malonylglucoside	OH	H	H

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ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Table I. (Continued). Proposed Identity of polyphenolic compounds found in hop extracts via HPLC-ESI-MS; letters A, B, C, D, E, F, G, H, and I refer to structures in Figure 2

Fraction 4						
Qucertin-3-O-(6”-O-malonylglucoside) (C)	548.9	506.9, 505.9, 504.9, 296.9	malonylglucoside	OH	OH	H
Kaempferol-3-O-rutinoside (C)	593.0	566.9, 394.0, 393.0, 362.9	rutinoside	OH	H	H
Kaempferol-3-O-(6”-O-oxalylglucoside) (C)	519.0	502.9, 392.7, 332.9, 286.9, 208.8	oxalylglucoside	OH	H	H
Unknown (715+2CH_2)	743.1	620.9, 417.0, 373.0, 371.9, 370.9, 363.0, 209.8, 208.8				
Unknown	371.0	279.1, 210.0, 209.0, 165.0				
Unknown	393.2	379.2, 371.1, 363.4, 349.2, 335.2, 209.9, 209.1				
Humulinone	377.0	333.2, 223.0, 195.0				
Fraction 5						
Unknown	295.1	216.9				
Unknown	234.0	216.8, 162.9, 145.0				
8-prenylnarigenin (H)	339.2	265.0, 264.1, 250.0, 249.0, 216.9	H	H	H	
Kaempferol-3-O-isohexenoyl (C)	383.2	285.1, 216.9	isohexenoyl	OH	H	H
Unknown	427.2	395.1, 351.1, 337.2, 285.1				
Unknown	471.2	462.5, 380.2, 331.2, 216.8				

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Fraction 5						
Unknown	381.2	363.2, 341.2, 321.1				
Unknown	515.3	457.2, 425.3, 395.1, 389.1, 379.2, 342.3				
Quercetin-3-O-rutinoside	609.0	573.3	rutinoside	OH	OH	H
Quercetin-3-O-glucoside (C)	463.1	301.0, 300.0	glucoside	OH	OH	H
Kaempferol-3-O-rutinoside (C)	593.0	533.2, 413.1, 285.0	rutinoside	OH	H	H
kaempferol-3-O-glucoside (C)	447.1	285.0, 284.0	glucoside	OH	H	H
Unknown	371.2	281.0, 265.0, 251.1, 243.0, 210.0, 209.0				
Unknown	381.2	364.2, 363.2, 306.1, 305.1, 275.2, 190.9				
Kaempferol-3-O-(6”-O-malonylglucoside) (C)	533.2	385.1, 384.1, 383.1, 312.1, 297.1280.1, 220.9	malonylglucoside	OH	H	H
Unknown	369.1	143.0				
Unknown	395.2	379.1, 378.4, 377.2, 319.1, 265.2				
Cohumulinone	363.2	249.0, 209.0, 141.0				
Unknown	317.2	248.0, 209.1, 205.0				

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Table I. (Continued). Proposed Identity of polyphenolic compounds found in hop extracts via HPLC-ESI-MS; letters A, B, C, D, E, F, G, H, and I refer to structures in Figure 2

Fraction 5						
Humulinone	377.2	365.3, 293.1, 263.1, 223.0				
Colupulone	399.3	330.1, 305.0, 287.1, 141.0				
Fraction 6						
Unknown	327.2	239.0, 229.0, 211.0				
Unknown	603.3	489.3, 465.2				
Unknown	329.2	249.0, 229.0, 211.0				
Dihydrohumulone	363.2	294.1				
Cohumulinone	363.2	353.1, 345.2, 249.0, 209.0, 140.9				
Humulinone	377.2	309.1, 308.1,				
Unknown (377+H_2O)	395.2	331.1, 317.2, 263.0, 248.0				
Humulone	361.2	347.2, 297.2, 265.1, 263.0				
Unknown	365.3	321.2, 285.2, 284.3, 283.2				
Colupulone	399.3	330.2,				
Fraction 7						
8-prenylnarigenin (H)	339.2	261.8, 210.8	H	H	H	
Unknown	383.2	218.8				

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Fraction 7						
Unknown	427.2	509.2, 275.0, 232.9				
Unknown	471.3	553.3, 283.1, 232.9, 166.8				
Unknown	515.3	597.3, 379.2,				
Unknown	559.3	641.3,				
Unknown	603.4	685.4, 574.6, 440.9, 411.2, 393.3, 269.2, 252.7				
Unknown	647.4	729.3, 550.0, 535.1, 232.9, 216.8				
Unknown	691.4	773.4, 675.2, 593.1, 561.1, 401.1, 396.2, 381.2, 232.9				
Dihydrohumulone/ cohumulinone	363.2	294.1, 201.9				
Dihydrohumulone/ cohumulinoe	363.2	249.1, 209.4				
Humulinone	377.2	309.1, 308.1, 248.9				
Xanthohumol	353.2	233.0				

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Table I. (Continued). Proposed Identity of polyphenolic compounds found in hop extracts via HPLC-ESI-MS; letters A, B, C, D, E, F, G, H, and I refer to structures in Figure 2

Fraction 7						
Humulone	361.2	347.2, 297.2, 263.0				
Colupulone	399.3	330.2				
Fraction 8						
Unknown	367.0	232.8, 176.8				
Unknown (367+O)	383.0	348.9				
Unknown	397.1	277.9				
xanthohumol	353.1	299.0, 162.9				
Unknown	427.1	293.9				
Unknown	339.1	162.8				
Unknown (377+H_2O)						
Humulone	361.2	347.2, 297.2, 265.1, 263.0				

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Conclusion

While this work is still preliminary and many compounds in the extract remain unresolved, the findings shed some light as to which compounds elicit higher antioxidant responses via the DPPH• radical quenching assay. Fractions 2 and 3 scored equally as high in antioxidant potential (78% and 81% respectively) and were high in total polyphenols and flavanoids. Given the general conviction that polyphenols and flavanoids are strong antioxidants, this was not surprising. However, it is interesting that despite measuring low in total polyphenols and low in flavanoids, fraction 4 was classified as being equally as high in antioxidant potential (79%) by the DPPH• radical quenching assay. Moreover it is interesting that fractions 6, 7 and 8, which were found to be high in hop bittering acids and their derivatives, showed relatively weak DPPH• antiradical activity. This finding conflicts with results from past experiments that implied hop bittering acids contribute significant antioxidant/antiradical potential to lager beer (*21*). It is our hope that continued investigation into the antioxidant nature of hop derived polyhenols in this manner will help shed light on the role of polyphenol s in beer flavor stability.

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Chapter 15

Tequila Processing and Flavor

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The alcoholic beverage tequila has gained popularity all around the world, due to its disctinctive flavor and aorma. Such characteristics are the result of a process that has been developed over almost five hudred years. Not much is known on the formation of flavor during the different processing steps performed to obtain tequila in its different types, and much more research is needed in order to better understand, improve and control the flavor of tequila. This chapter discusses the knowledge to date on the volatile flavor compounds that are originated during tequila production.

Introduction

The fermented juice from the agave plant has been consumed by indigenous people in America for a long time. But it was until the sixteenth century that distillation was introduced from Spain, and the beverage began to be distilled (*1*) giving birth to a clear high-alcohol containing spirit: mezcal. As mezcal was gaining popularity across central Mexico between the eighteenth and nineteenth centuries, people from a small town named Tequila in the state of Jalisco, Mexico, improved the production process, obtaining a better quality product that was latter called tequila because of its place of origin. One of the secrets of the people from Tequila was the use of a certain kind of agave: the blue variety, since that place has excellent climatological and land conditions for its growth (*2*).

In Flavor Chemistry of Wine and Other Alcoholic Beverages; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

On may 27, 1997, tequila spirit was granted with the Protected Designation of Origin by the European Union, which means that only products originating in a certain region are allowed in commerce identified as such. This status is also protected by the North American Free Trade Agreement (NAFTA) between United States, Canada and Mexico (*3*). Since then, tequila has become one of the most popular spirits in the world, highly increasing its sales due to its distinctive flavor and aroma (*4*).

There has been some information published regarding chemical composition of tequila flavor (Table I), although much more is needed for a better scientific understanding. By using either liquid-liquid continuous and batch extraction, or simultaneous distillation-extraction, all coupled to mass spectrometry analysis, researchers identified up to 129 different compounds, and the liquid-liquid batch extractive method showed the best recovery (*5*). Other authors utilized dicloromethane extraction and Kuderna-Danish concentration, followed by gas chromatography coupled to mass spectrometry, flame ionization, and sulfur chemiluminiscence detections and aroma extract dilution analysis. They identified 175 compounds in tequila (*6*). The application of more modern and sophisticated tecniques of analysis such as solid phase microextraction and comprehensive two-dimensional gas chromatograpy mass spectrometry only yielded 113 chemical compounds for a tequila spirit, probably due to a lack of extraction optimization (*7*). In the following text, the published information at hand will be arranged and explained according to the different operations that occur during tequila production: agave harvesting, “piñas” baking and syrup extraction, fermentation, distillation, aging, and quality-authenticity control.

Agave Harvesting

There are about 310 different agave species in the American continent, but only few of them are used for production of fermented beverages (*2*). By law, *Agave tequilana* Weber (blue variety) is the only agave plant allowed for the production of tequila (*8*). It takes 7-9 years for the agave to be ready for industrial processing. At age 6-8 years, agaves begin to grow a flower stalk, which is immediately cut so the center of the plant fattens and ripens for an extra 7 months before leaves are eliminated, resulting in a bulbous round form called piña, with an average 27% content of reducing sugars (*8*). It is in this stage that many flavor precursors begin to accumulate in the plant.

Sugars, mainly fructose, starch, and inulin which is a fructose polymer, are present in high proportions especially during low-rain years (*9*). Sugars are the main substrate for ethanol production by fermentative bacteria. Other important compounds are present, such as lipids. It has been reported that the amount and kind of lipids vary between different agave species, and include free fatty acids, β-sitosterol, and groups of mono-, di-, and triacylglycerols, with total concentration ranging from 459 to 992 μg/g of agave. Fatty acids are present from C10 to C24, being C18, C18:1, C18:2 and C18:3 the most abundant ones. Acylglycerols present in agave give rise to free fatty acids and their corresponding ethyl esters in the beverage (*10*). Some free fatty acids from the agave can remain through all the processing steps and make it to the final distilled beverage, giving specific odor and taste profiles to each drink, with oily and musty notes (*6*, *11*).

22 terpenoid compounds have been identified in tequila spirit and it is believed that they come from the agave cactus. The most abundant terpene compounds are linalool, α-terpineol, and *trans, trans*-α-farnesol (*6*) which give floral, sweet, citrus, woody, resinous notes (*6*, *12*, *13*). Carotenoids in agave can lead to degradation products like β-cyclocitral and β-damascenone (*14*, *15*) responsible of the camphoraceous and fruity,winey,woody notes respectively (*6*, *12*, *13*). Some phenols like *p*-cresol and 4-ethyl phenol are also breakdown products of phenolic acids which are originally present in the plant, giving a characteristic phenolic aroma (*6*, *12*, *13*). Many other chemical compounds present in the agave may serve as precursors and lead to different flavor products during the many stages of tequila processing, but more comprehensive research is still needed.

"Piñas" Baking and Syrup Extraction

The mature and leavesless agave, so called "piña", is taken to the factory and cut in halves, quarters, or more, in order to make it easier for oven (48 h) or autoclave (12 h) baking at 106-116°C. This thermal treatment has the objective of hydrolyzing complex sugars like inulin and starch, to obtain glucose and fructose for an easier fermentation (*9*). As discussed before, sugars result mainly in ethanol formation, although many other flavor compounds arise from the fermentation of this substrate. Resulting from the thermal treatment of the piñas, many flavor compounds are formed, mainly Maillard-related such as furans, pyrans, aldehydes, nitrogen and sulfur compounds. The most abundant Maillard compounds are methyl-2-furoate, 2,3-dihydroxy-3,5-dihydro-6-methyl-4(H)-pyran-4-one and 5-hydroxymethylfurfural (*16*), which are responsible of burnt and caramel-like flavor (*6*, *12*, *13*). Pyrazines are also an important group of chemical compounds derived from Maillard reactions. Most abundant pyrazines found are 2,5-dimethylpyrazine and trimethylpyrazine (*6*) with roasty and nutty aromas (*6*, *12*, *13*).

Other thermally-related breakdown products arise during the baking step. Free fatty acids of short- and long-carbon chain have been found in baked piñas probably due to hydrolisys of acylglycerols (*16*) giving sweaty, oily and musty notes (*6*, *12*, *13*). β-cyclocitral and β-damascenone are likely degradation products of carotenoids (*14*, *15*), while 4-methyl-5-(2-hydroxyethyl)-thiazole is a breakdown product of the amino acid thiamine (*17*). Phenols like *p*-cresol and 4-ethyl phenol are a breakdown product of phenolic acids.

All these compounds are in a way or another transferred to the final spirit, and have been reported as powerful odorants responsible of an important part of the tequila flavor. There have been some attempts to use enzymes to improve inulin and starch hydrolysis, so sugar and ethanol yield is increased (*18*), but producers have not agreed to eliminate the baking step since it is the responsible of the formation of very important flavor compounds.

Once the piñas are baked, they are taken to a shredding mill and a crusher where they release all the syrup containing high concentration of sugars and the majority of the flavor compounds formed so far. The resulting agave mash is often washed off in oder to improve sugar extraction (*9*). It is in this stage where the two main types of tequila take their road: 100% agave and “mixto”. The highest quality tequila 100% agave must be obtained from the fermentation of only syrup extracted from piñas of *Agave tequilana* Weber and may contain some added water. Mixto can use up to 49% of syrup from other origin, such as sugar cane (*8*), consecuently lacking much of the flavor compounds present in the baked agave syrup. This dilution renders a lower quality flavor, so mixto tequila is therefore sold cheaper.

Fermentation

There is no doubt that fermentation is the most important and complicated stage of tequila processing. It is possible that most of the compounds shown in Table I are formed during fermentation. 100% agave or mixto syrups are diluted with water to reach 12-14°Brix (80-100 g/L of sugar). Fermentation takes place in thermostatized tanks at 30°C, although some processes are carried out at room temperature, which could be variable depending on the season of the year (*19*). Fermentation depends entirely of the metabolism of yeasts and in less extent of lactic and acetic acid bacteria. Many strains of yeasts have been found in agave musts, being *Saccharomyces cerevisiae* and *Kloeckera africana* the most important ones (*20*). Yeasts metabolize carbohydrates, amino acids, fatty acids and other organic compounds, transforming them into ethanol, glycerol, carbon dioxide, and in a less extent into aldehydes, ketones, higher alcohols, organic acids, and esters, which are called “fermentation by-products” or “congeners” (*21*). Higher alcohols, also called “fusel alcohols” because of their malty and burnt flavor, are formed by degradation of amino acids via keto acids (2-oxo acids). The most important ones are 1-propanol, 2-methyl-1-propanol, 2-methyl-butanol, 3-methyl-butanol, and 2-phenylethanol, the later having a rose-like aroma (*22–26*). Synthesis of fatty acids inside the yeast cell forms mainly saturated straight-chain fatty acids with an even number of 4 to 18 carbon atoms, and the appearance of low levels of fatty acids with odd carbon numbers and unsaturations depends on the fermentation conditions (*23*). Fatty acids can combine with alcohols to form esters. Esters are the largest group of flavor compounds (Table I) and their contributions to flavor are of great importance with mostly pleasant notes (*6*, *12*, *13*), especially the low boiling point ethyl esters and acetates (*27*). Ethyl hexadecanoate and ethyl octadecanoate are the most abundant esters, and the amounts of ethyl esters vary according to the type of tequila (*28*).

It is known that different yeast strains produce different flavor compounds (*29–31*), being the reason why each industry uses a secret combination of strains in order to achieve the characteristic brand profile (*9*). This is perhaps more important than the type or amount of agave used. Other factors influencing flavor production during fermentation are time, temperature and carbon/nitrogen ratio (*29*). Tequila produced with a slow fermentation process (24 hours or more) is richer in flavor and aroma, in comparison with the one produced in a fast process

(18 to 20 hours) (*29*). A processing temperature of 35°C produces more volatile compounds than 30°C (*29*, *32*). Also, it has been observed that supplementation with a nitrogen source changes flavor formation by yeasts during fermentation, although the effect is different depending on the nitrogen source used (*33*). By adding a mixture of 20 amino acids, *Kloeckera africana* strain K1 is able to produce and tolerate higher ethanol concentrations, while the production of some esters, alcohols, acetaldehyde and α-terpineol is increased (*34*). When using *Saccharomyces cerevisieae* in a must supplemented with sodium sulfate and amino acids, the concentration of amyl alcohols and isobutanol decrease, while propanol an acetaldehyde increase (*33*).

Distillation

Once the fermentation is over, and alcohol content reaches about 15% v/v, it is time for distillation. The fermented mash is heated in copper or stainless steel kettles at 78-80°C so evaporation of alcohol is achieved. Vapors are condensated in cooled coils and a distillate is collected. First distillate reaches an alcohol concentration of ~25% v/v, and needs a second distillation also called rectification, in order to reach ~55% v/v of ethanol. This liquid is then adjusted with water to 38-40% v/v alcohol and bottled to be sold as silver type tequila (*9*, *19*). Since the majority of flavor compounds in tequila are volatile, then they are evaporated along with ethanol during distillation. It is possible to separate different fractions of volatiles or "cuts" during distillation. The head cut contains highly volatile compounds like acetaldehyde and ethyl acetate, whereas the tail cut has higher boiling point chemicals such as ethyl esters of long-chained fatty acids. Since the falvor notes of both fractions are commonly undesirable, they can be separated from the heart cut which is characterized by more pleasant flavor compounds (*21*). Metanol is obtained in the tail, despite its low boiling point. Ethyl lactate, acetic acid and furfural are also distilled in the tail fraction. Isobutyl and isoamyl alcohols behave as head products, n-propyl alcohol is found in the heart, and phenetyl alcohol exhibits a tail product behavior (*35*).

The use of copper kettles affects the final flavor of tequila, by enhancing the development of fruity and flowery notes (*35*). It has ben reported that use of copper pots for distillation increases the copper content in the final beverage. Copper could contribute to the catalytic destruction of sulphur-containing off-flavor compounds that come mainly from the agave plant, perhaps giving better taste and aroma to the beverage (*36–38*) although this has never been proved in tequila. Nowadays, boiling in copper kettles has been limited since a maximum copper concentration has been set for tequila (*8*). Copper concentration has also been associated to high concentration of acids in distilled beverages (*39*).

Heat also plays an important role on flavor generation during ditillation. Because of this it is possible that some breakdown reactions take place, and formation of aldehydes, ketones, furans, sulfur compounds, pyrazines, and phenols occur (*6*).

Aging

There are five types of tequila according to current regulations. The silver type, which is the rectified distillate adjusted with water to commercial ethanol content; the gold or young tequila, consisting of silver type added with color and flavor; the "reposado" type, which is silver tequila aged for at least 2 months in oak casks and may be added with color and flavor; the aged kind, matured for a minimum of one year in oak recipients; and the extra aged one, aged for at least three years in oak casks (*40*, *41*). All of them could be 100% agave or mixto. Aging for more of 4 years is not a common practice, since it has been observed that it overwhelms the distinctive earthy and vegetal agave flavor notes.

The raw pungent flavor of tequila mellows with aging. Aldehydes evaporate and/or form acetals. Alcohol also slowly escapes, and the aroma gets more intense, complex and concentrated. By storing in wooden casks, volatile compounds such as vanillin, guaiacol, eugenol, cresol and other phenolics migrate from the wood to the distillate, up-rounding the flavor (*42*). Ethyl esters are not only formed during fermentation, but also during aging. It has been reported that esters may be formed subsequently during the aging process by esterification of fatty acids with ethanol at high concentrations (*6*). This is the main reason why aged and extra-aged tequilas show the highest amounts of even carbon number ethyl esters from C:6 to C:18 (*28*). In general, many more volatile compounds have been found in aged and reposado tequilas when compared to the silver type (*43*).

Quality-Authenticity Control

High levels of 1-butanol and 2-butanol are indicators of a possible utilization of spoiled raw materials. Other substances like acrolein, diacetyl, allyl alcohol, acetic acid, and acetaldehyde are a result of uncontrolled microbiological activity or a poor distillation technique, and depending on their concentrations may cause off-flavor (*22–25*, *44*). Ethyl acetate, mainly produced by esterification of acetic acid, contributes significantly to a solvent-like nail polish off-flavor at high levels (*27*).

Also, adulteration with other source of alcohol like grain spirits, and mixing of different types of tequilas is a violation of the standard. Due to increasing international sales of tequila, fakes are becoming common and there is a need for authenticity tests. Many components of the beverage have been studied as possible originality markers, such as metals and minerals (*45*, *46*), anions (*47*), and others. Flavor compounds have been poorly studied as indicators for tequila genuineness. Variations in the methanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol concentrations have been observed when comparing 100% agave and mixto tequilas, with lower concentrations in the mixto category (*47*).

The official standard has set wide ranges for the concentrations of volatile compounds, and are almost identical for the four tequila types (*8*). More information is needed in order to identify the original flavor of the protected name tequila, as well as to check the labeled category.

Table I. Compilation of flavor compounds found in tequila

	Retention Index		*Compound*	*Identification*	*Extraction*	*Odor descriptor*	*Max dilution*[a]	*Threshold*	*References*
	DB 5	*DB WAX*							
Acetals									
			acetaldehyde methyl ethyl acetal	MS	LLB, LLC				(*6*, *13*)
			formaldehyde diethyl acetal	MS					(*6*)
	730	1096	acetaldehyde diethyl acetal	MS, RI, ST	LLB, LLC	fruity		4 µg/Kg	(*6*, *12*, *13*)
			acetaldehyde propylene glycol acetal	MS		medium strength odor			(*6*)
			acetaldehyde 2,3-butanediol acetal	MS					(*6*)
	817		propanal diethyl acetal	MS, ST	LLB, LLC				(*6*, *13*)
			acetaldehyde ethyl propyl acetal	MS					(*6*)
			isobutyraldehyde diethyl acetal (and propan-2-ol)	MS, RI	LLB, LLC				(*6*, *13*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
		acetaldehyde ethyl isobutyl acetal	MS					(*6*)
		butanal diethyl acetal	MS					(*6*)
		2-methylbutanal propylene glycol acetal?	MS					(*6*)
		acetaldehyde ethyl butyl acetal	MS					(*6*)
		2-methylbutanal diethyl acetal	MS					(*6*)
		3-methylbutanal diethyl acetal	MS	LLC				(*6*, *13*)
		acetaldehyde ethyl 2-methylbutyl acetal	MS					(*6*)
		acetaldehyde ethyl 3-methylbutyl acetal	MS					(*6*)
		acetaldehyde ethyl pentyl acetal	MS					(*6*)

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution[a]***	***Threshold***	***References***
DB 5	***DB WAX***							
		acetaldehyde 2-methylpropyl 2-methylbutyl acetal	MS					(*6*)
		acetaldehyde 2-methylpropyl 3-methylbutyl acetal	MS					(*6*)
		unidentified acetal	MS					(*6*)
		acetaldehyde diisoamyl acetal	MS		coarse character			(*6*, *13*)
		phenylacetaldehyde diethyl acetal	MS, RI		green, foliage, floral, rosy, earthy, mushroom			(*6*)
		acetaldehyde ethyl phenylethyl acetal	MS, RI, GCO		sharp, floral, fruity	50		(*6*)
		n-hexanal diethyl acetal	MS, RI	LLB	cognac, pear, floral, hyacinth, apple, fruity			(*6*, *13*)
1075		1,1,3-triethoxi propane	MS	LLB				(*13*)
Acids								

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Table I. (Continued). Compilation of flavor compounds found in tequila

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
600	1477	acetic acid	MS, RI, ST	LLB, LLC, SDE	vinegar-like, pungent		100 mg/Kg	(*6*, *13*, *21*)
		propionic acid	MS, RI, ST	LLB, LLC	rancid odor		5 mg/Kg	(*12*, *13*)
1215	1588	2-methylpropanoic acid	MS, RI, GCO	LLC	fruity, sweaty, rancid butter	50	10 µg/Kg	(*6*, *12*, *13*)
820	1628	butyric acid	MS, RI	LLB, LLC, SDE	buttery		240 µg/Kg	(*13*, *21*)
	1672	2-methylbutyric acid	MS, RI, GCO		fruity, sweaty, acidic, dirty, cheesey with a fermented nuance	800	10 µg/Kg	(*6*, *12*)
911	1698	pentanoic acid	MS, RI	LLB, LLC	similar to butyric acid		940 µg/Kg	(*6*, *12*, *13*)
877		isovaleric acid	MS, RI, ST	LLB, LLC, SDE	disagreeable,rancid,cheese odor		190 µg/Kg	(*12*, *13*)

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
1019	1797	hexanoic acid	MS, RI, ST	LLB, LLC, SDE	rancid, fatty		93 µg/Kg	(*6*, *13*, *21*)
	1900	heptanoic acid	MS, RI	LLB, LLC	disagreable rancid,fatty odor		640 µg/Kg	(*12*, *13*)
1279	2065	octanoic acid	MS, RI, GCO, ST	LLB, LLC, SDE	fatty acid, dry, dairy, oily, soapy	50	910 µg/Kg	(*6*, *13*, *21*)
1373		decanoic acid	MS, RI	LLB, LLC, SDE	fatty, citrus		2.2 mg/Kg	(*6*, *13*, *21*)
		decanoic acid + ethyl hexadec-9-enoate	GCO		fatty acid, dry, woody	3200		(*6*)
		unidentified C10 acid	GCO		powder, fatty, sharp	800		(*6*)
1568	2517	dodecanoic acid	MS, RI, GCO, ST	LLB, LLC, SDE	fatty acid	400	5 mg/Kg	(*6*, *12*, *13*)
1720	2724	tetradecanoic acid	MS, RI, ST	LLB, LLC, SDE	faint, waxy, oily odor		10 mg/Kg	(*6*, *12*, *13*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

	Retention Index		*Compound*	*Identification*	*Extraction*	*Odor descriptor*	*Max dilution*[a]	*Threshold*	*References*
	DB 5	*DB WAX*							
	1984	2940	hexadecanoic acid	MS, RI, ST	LLB, LLC, SDE	virtually odorless		10 mg/Kg	(*6*, *12*, *13*)
	2023		hexadecenoic acid	MS, RI					(*6*)
	1274	2585	phenylacetic acid	GCO		pungent, floral, honey	800	1 mg/Kg	(*6*, *12*)
Alcohols									
	668	936	ethanol	MS, RI, ST	LLB, LLC, SDE	alcoholic		8 µg/Kg	(*6*, *13*, *21*)
	536		propanol	MS, RI, ST	LLB, LLC, SDE	alcoholic		5.7 mg/Kg	(*6*, *13*, *21*)
	647	1103	isobutyl alcohol	MS, RI, GCO, ST	LLB, LLC, SDE	sweet, chemical, wine-like odor	800	360 µg/Kg	(*6*, *12*, *13*)
	730	1116	pentan-2-ol	MS, RI		mild green, fusel-oil odor			(*6*, *12*)

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
675	1142	butanol	MS, RI, GCO, ST	LLB, LLC, SDE	sweet, fusel	50	500 µg/Kg	(*6*, *13*, *21*)
755	1208	2-methylbutanol	MS, RI, ST	LLB, LLC, SDE	malty		40 mg/Kg	(*6*, *13*, *21*)
738	1208	3-methylbutanol	MS, RI, GCO, ST	LLB, LLC, SDE	sweet, fruity, fusel	6400	250 µg/Kg	(*6*, *13*, *21*)
		3-methyl-2-butanol	MS, RI	LLB, LLC, SDE	fruity, fresh odor		410 µg/Kg	(*12*, *13*)
		3-methylbut-3-en-1-ol	MS, RI	LLB, LLC	sweet, fruity			(*6*, *6*, *13*)
768	1244	pentanol	MS, RI, ST	LLB, LLC, SDE	fusel-like sweet and pleasant odor		1.6 mg/Kg	(*6*, *12*, *13*)
		pent-4-en-1-ol	MS	LLB, LLC, SDE				(*6*, *13*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
854	1313	3-methylpentan-1-ol	MS, RI	LLB, LLC	pungent, fusel, cognac and wine, cocoa, with green fruity undernotes		830 µg/Kg	(*6*, *6*, *13*)
846	1301	4-methylpentan-1-ol	MS, RI	LLB, LLC	nutty			(*6*, *6*, *13*)
851	1362	hexanol	MS, RI, ST	LLB, LLC, SDE	green, flowery		200 µg/Kg	(*6*, *13*, *21*)
991	1368	octan-3-ol	MS, RI, ST	LLB, LLC	sweet, oily, nutty, warm, herbaceous		18 µg/Kg	(*6*, *12*, *13*)
984	1332	2-octanol	MS, RI	LLB, LLC	disagreeable, but aromatic odor		7.8 µg/Kg	(*12*, *13*)
977	1438	oct-1-en-3-ol	MS, RI, GCO		mushroom, earthy, sweet, with a strong, herbaceous note reminiscent of lavender-lavandin, rose and hay	200	14 µg/Kg	(*6*, *12*)
877	1225	heptanol	MS, RI		fragrant, woody, heavy, oily, faint, aromatic, fatty oddor and a pungent, spicy taste		3 µg/Kg	(*6*, *12*)

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
981	1388	octanol	MS, RI, ST	LLB, LLC	fresh, orange-rose odor, quite sweet and a oily, sweet, slightly herbaceous taste		42 µg/Kg	(*6*, *12*, *13*)
1263	1765	decanol	MS, RI, ST	LLB, LLC	floral odor resembling orange flowers and a slight, characteristic fatty taste		6 µg/Kg	(*6*, *12*, *13*)
1118	1925	2-phenylethyl alcohol	MS, RI, GCO, ST	LLB, LLC, SDE	floral, rose-like odor and an initially slightly bitter taste, then sweet and reminiscent of peach	6400	1.2 mg/Kg	(*6*, *12*, *13*)
1577	1972	dodecanol	MS, RI, ST	LLB, SDE	fatty odor, fatty, waxy flavor		73 µg/Kg	(*6*, *12*, *13*)
		tetradecanol	MS, RI, ST	LLB, SDE				(*6*, *13*)
1870	2218	hexadecanol	MS, RI, ST	LLB, SDE	odorless			(*6*, *12*, *13*)
	1300	cyclopentanol	MS, RI	LLB, LLC				(*13*)
886	1403	cyclohexanol	MS, RI	LLB, LLC				(*13*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

	Retention Index		*Compound*	*Identification*	*Extraction*	*Odor descriptor*	*Max dilution*[a]	*Threshold*	*References*
	DB 5	*DB WAX*							
			sec-nonyl-alcohol	MS	LLB, LLC				(*13*)
	1039	1865	benzyl alcohol	MS, RI, ST	LLC	fruity odor and a slightly pungent, sweet taste		1.2 µg/Kg	(*12*, *13*)
			benzene propanol	MS	LLB, LLC	sweet, hyacinth-mignonette odor. sweet and pungent taste suggetive of apricot		20 mg/Kg	(*12*, *13*)
Aldehydes									
	427	714	acetaldehyde	MS, RI, CGO	LLB, LLC	dhemical, sharp, penetrating, ethereal odor	800	0.7 µg/Kg	(*6*, *12*, *13*)
	506	571	propanal	MS, RI	LLB, LLC	sharp and pungent odor similar to acetaldehyde		9.5 µg/Kg	(*12*, *13*)
	662	821	isobutyraldehyde	MS, RI, CGO	LLB, LLC	sweet, caramel, sharp, pugent	800	0.4 µg/Kg	(*6*, *12*, *13*)

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
		2-methylbutanal and 3-methylbutanal	MS, RI		powerful, choking , acrid, pungent, apple-like			(*6*, *12*)
623	1041	but-2-enal	MS					(*6*)
996	1525	benzaldehyde	MS, RI, ST	LLB, SDE	almond		100 µg/Kg	(*6*, *13*, *21*)
		α-cyclocitral	MS	LLB, LLC	camphoraceous odor for a 50/50 mixture of isomers			(*12*, *13*)
1220	1623	β-cyclocitral	MS, RI		camphoraceous odor for a 50/50 mixture of isomers			(*6*, *12*)
1049	1609	phenylacetaldehyde	MS, RI, CGO	LLB, LLC	floral, sharp, harsh, green odor reminiscent of hyacinth on dilution	200	4 µg/Kg	(*6*, *12*, *13*)
1819	2120	hexadecanal	MS, RI		cardboard			(*6*, *12*)
654	912	isovaleraldehyde	GCO		sweet, cocoa, chocolate, acrid, pungent, apple-like odor	6400		(*6*, *12*)
		trans-2-nonenal	GCO		dry, leafy, green, cucumber, aldehydic, fatty with a citrus nuance	100		(*6*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

	Retention Index		*Compound*	*Identification*	*Extraction*	*Odor descriptor*	*Max dilution*^{a}	*Threshold*	*References*
	DB 5	*DB WAX*							
			3-ethoxy propanal	MS	LLB, LLC				(*13*)
	593	932	butanal	MS, RI, ST	LLB	pungent		19 µg/Kg	(*12*, *13*)
Esters									
		856	methyl acetate	MS, RI		pleasant, fruity odor and slightly bitter flavor		1.5 mg/Kg	(*6*, *12*)
	628	907	ethyl acetate	MS, RI, ST	LLB, LLC	solvent-like, nail polish		5 µg/Kg	(*6*, *13*, *21*)
	709	950	ethyl propionate	MS, RI, GCO, ST	LLB, LLC	sweet, butterscorth, fruity	800	9 µg/Kg	(*6*, *13*)
	762	965	ethyl isobutyrate	MS, RI	LLB, LLC	fruity aromatic odor		0.01 µg/Kg	(*6*, *12*, *13*)

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
712	969	propyl acetate	MS, RI		fruity (pear-raspberry) odor with a pleasant, bittersweet flavor reminiscent of pear on dilution		2.7 mg/Kg	(*6*, *12*)
812	1105	butyl acetate	MS, RI		strong, fruity odor; burning and then sweet taste reminiscent of pineapple		10 µg/Kg	(*6*, *12*)
800	1022	ethyl butyrate	MS, RI, GCO	LLB, LLC	fruity, banana	50	0.1 µg/Kg	(*6*, *13*, *21*)
		ethyl 2-methylbutyrate	MS, RI		powerful, green-fruty, apple-like odor		0.01 µg/Kg	(*6*, *12*)
840	1070	ethyl isovalerate	MS, RI		strong, fruity, vinous, apple-like odor on dilution		0.01 µg/Kg	(*6*, *12*)
		isoamyl formate	MS, RI		plum, fruity characteristic odor suggestive of black currant, with a corresponding sweet taste		15 mg/Kg	(*6*, *12*)
898	1120	ethyl valerate	MS, RI		fruity odor siggestive of apple		1.5 µg/Kg	(*6*, *12*)
915		amyl acetate	MS, RI		fruity			(*6*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
997	1224	ethyl hexanoate	MS, RI, GCO, ST, SPME	LLB, LLC, SDE	fruity, apple, banana, violet	100	0.3 µg/Kg	(*6*, *13*, *21*, *28*)
1111		ethyl 2,4-hexadienoate	MS		warm, fruity odor		30 mg/Kg	(*6*, *12*)
	1242	ethyl pyruvate	MS		vegetable, caramel odor		60 mg/Kg	(*6*, *12*)
1095		ethyl heptanoate	MS, RI		fruity odor reminiscent of cognac with a corresponding taste		2 µg/Kg	(*6*, *12*)
1010	1358	ethyl lactate	MS, RI	LLB, LLC, SDE	ligth ethereal, buttery odor		50 mg/Kg	(*6*, *12*, *13*, *21*)
1125	1378	methyl octanoate	MS, RI		powerful, winy, fruity and orange-like odor and an oily, somewhat orange taste		200 µg/Kg	(*6*, *12*)
1195	1422	ethyl octanoate	MS, RI, ST, SPME	LLB, LLC, SDE	pineapple, pear		5 µg/Kg	(*6*, *13*, *21*, *28*)

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
1297	1528	ethyl nonanoate	MS, RI		slightly fatty, oily, nutty, fruity, odor reminiscent of cognac with a rosy- fruity note		5 mg/Kg	(*6*, *12*)
1078		ethyl 2-hydroxy-4-methylpentanoate	MS					(*6*)
		2-methylpropyl octanoate	MS, RI		fruity			(*6*)
		3-methylbutyl lactate	MS, RI		creamy, nutty			(*6*)
1326	1590	methyl decanoate	MS, RI		oily wine fruity floral			(*6*)
1397	1630	ethyl decanoate	MS, RI, GCO, ST	LLB, LLC, SDE, SPME	sweet, dairy, floral, fatty	50	8 µg/Kg	(*6*, *13*, *21*, *28*)
		3-methylbutyl octanoate	MS, RI		fruity		20 mg/Kg	(*6*, *12*)
		ethyl succinate	MS, RI, ST	LLB, LLC, SDE				(*13*)
1191	1690	diethyl succinate	MS, RI		faint, pleasant odor		10 mg/Kg	(*6*, *12*, *21*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
		ethyl dec-9-enoate	MS		fruity, fatty			(*16*)
1190		methyl salicylate	MS, RI, ST	LLB, LLC, SDE	minty, spicy, sweet, wintergreen-like odor		40 µg/Kg	(*6*, *12*, *13*)
1244	1724	ethyl phenylacetate	MS, RI	LLC	strong, sweet odor siggestive of honey and a bittersweet flavor		650 µg/Kg	(*6*, *12*, *13*)
1526	1813	methyl dodecanoate	MS, RI		fatty, floral odor reminiscent of wine		20 mg/Kg	(*6*, *12*)
1265	1803	phenylethyl acetate	MS, RI	LLB, LLC, SDE	floral odor reminiscent of rose with honey-like undertone and a sweet, fruit-like taste reminiscent of raspberry		3 mg/Kg	(*6*, *12*, *13*)
1595	1822	ethyl dodecanoate	MS, RI	LLB, LLC, SDE, SPME	floral, fruity		50 mg/Kg	(*6*, *12*, *13*, *21*, *28*)
		isoamyl decanoate	MS, RI	LLB, LLC, SDE	waxy, banana, fruity, sweet, cognac, green			(*6*, *6*, *28*)

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
996	1877	phenylethyl isobutyrate	MS, RI		fruity odor and a bittersweet taste reminiscent of unripe plum, pineapple and banana		2.5 mg/Kg	(*6*, *12*)
	1897	ethyl 3-phenylpropionate	MS, RI	LLB, LLC, SDE	ethereal, rum, fruity, floral		17 µg/Kg	(*6*, *12*, *13*)
		ethyl dodecenoate	MS					(*6*)
		phenylpropyl acetate	MS, RI		floral, spicy odor reminiscent of phenylpropyl alcohol and of geranyl acetate eith a bittersweet, burning flavor suggestive of currant		10 mg/Kg	(*6*, *12*)
1439	1915	phenylethyl butyrate	MS, RI	LLB	rose-like fragrance and a sweet taste, suggestive of honey		25 mg/Kg	(*6*, *12*, *13*)
1793	2029	ethyl tetradecanoate	MS, RI	LLB, LLC, SDE, SPME	mild, waxy, soapy odor reminiscent of orris		4 mg/Kg	(*6*, *12*, *13*, *28*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
1993	2225	ethyl hexadecanoate	MS, RI	LLB, LLC, SPME	mild, waxy sweet		2 mg/Kg	(*6*, *12*, *13*, *28*)
		ethyl hexadec-9-enoate	MS					(*6*)
		phenylethyl octanoate	MS		mild, fruity, wine-like		10 mg/Kg	(*6*, *12*)
2179	2480	ethyl oleate	MS, RI	LLB	faint, floral note		130 mg/Kg	(*6*, *12*, *13*)
2159	2505	ethyl linoleate	MS, RI	LLB, LLC, SDE				(*6*, *13*)
2169	2596	ethyl linolenate	MS, RI	LLB, LLC, SDE				(*6*, *13*)
767	1005	isobutyl acetate	MS, RI	LLB	fruity (currant-pear), floral (hyacinth-rose) odor and a characterisitic ether-like, slightly bitter flavor		65 µg/Kg	(*12*, *13*)

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
877	1118	isoamyl acetate	MS, RI	LLB, LLC, SDE	fruity, banana, sweet, fragrant, powerful odor with a bittersweet taste reminiscent of pear		2 µg/Kg	(*12*, *13*)
		vethyl 2-hydroxyisoamilate	MS	LLB, LLC				(*13*)
		ethyl levulinate	MS, RI, ST	LLB, LLC	ethereal, fruity, green, sweet, pineapple, apple, rhubarb odor		40 mg/Kg	(*12*, *13*)
1210		methyl 4-methyl-benzoate	MS, RI	LLB, LLC	sweet, anisic, floral, ylang			(*12*, *13*)
1442		isoamyl benzoate	MS, RI	LLB	mild, sweet, fruit-like		25 mg/Kg	(*12*, *13*)
1990		ethyl palmitoleate	MS	LLB, LLC, SDE				(*13*)
1495	1986	phenethyl isovalerate	MS, RI	LLB, LLC, SDE	fruity (rose-like) odor and a bittersweet flavor, reminiscen of peach		2 mg/Kg	(*12*, *13*)
2194	2389	ethyl octadecanoate	MS, RI	LLB, SPME	virtually odorless			(*12*, *13*, *28*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

	Retention Index		*Compound*	*Identification*	*Extraction*	*Odor descriptor*	*Max dilution*[a]	*Threshold*	*References*
	DB 5	*DB WAX*							
			dioctyl adipate	MS, RI	LLB, LLC, SDE				(*13*)
Furans									
			2-methyltetrahydrofuran-3-one	MS, RI, ST	LLB, LLC, SDE	reminiscent of wintergreen		75 mg/Kg	(*6*, *12*, *13*)
			furfuryl ethyl ether	MS, GCO		sharp, chemical	200		(*6*)
			5-methylfurfuryl ethyl ether	MS					(*6*)
	832	1474	furfural	MS, RI, ST	LLB, LLC, SDE	smoky, almond		280 µg/Kg	(*6*, *13*, *21*)
	910	1475	2-acetylfuran	MS, RI, ST	LLB, LLC, SDE	coffe-like		10 mg/Kg	(*6*, *12*, *13*)
	991	1559	furfuryl acetate	MS, RI, ST	LLC	ethereal floral fruity odor			(*6*, *12*, *13*)

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
978	1560	5-methylfurfural	MS, RI, ST	LLB, LLC, SDE	sweet, spicy, warm odor with a sweet, caramel-like flavor		6 mg/Kg	(*6*, *12*, *13*)
		2-propionylfuran	MS, RI	LLB, LLC				(*6*, *13*)
983	1569	methyl 2-furoate	MS, RI		fruity odor similar to mushroom, fungus or tobacco with a sweet, tart, fruity taste that is quite heavy		20 mg/Kg	(*6*, *12*)
1056		ethyl 2-furoate	MS		ethyl benzoate, fruity, floral			(*6*)
866	1669	furfuryl alcohol	MS, RI	LLC	mild, warm, oily, "burnt" odor and a cooked sugar taste		1 mg/Kg	(*6*, *12*, *13*)
		2-methyl-2-vinyl-5-octadienyltetrahydrofuran	MS					(*6*)
1224		(hydroxymethyl)furfural	MS, RI					(*6*)
920		5-methyl-2-(3H)-furanone	MS, ST	LLB, LLC	sweet, herbaceous odor reminiscent of tobaco		100 mg/Kg	(*12*, *13*)
1039	1658	2-acetyl-5-methyl furan	MS	LLC	nutty		50 mg/Kg	(*12*, *13*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

	Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***^{a}	***Threshold***	***References***
	DB 5	***DB WAX***							
Ketones									
	593	983	diacetyl	MS, RI, GCO		butter	800	0.3 µg/Kg	(*6*, *21*)
	698	792	acetylpropionyl	MS, RI, GCO		similar to quinone,buttery diacetyl,fermented dairy and creamy	200	20 µg/Kg	(*6*, *12*)
	606	1138	pent-3-en-2-one	MS		fruity odor becoming pungent on storage		1.5 µg/Kg	(*6*, *12*)
			heptan-2-one	MS, RI		fruity,cinnamon,banana		1 µg/Kg	(*6*, *12*)
	767	1154	cyclopentanone	MS, RI	LLB, LLC, SDE	peppermint,musty,nutty		20 mg/Kg	(*6*, *12*, *13*)
		1194	3-methylcyclopentanone	MS					(*6*)
	974	1365	6-methylhept-5-en-2-one	MS, RI		citrus odor		50 µg/Kg	(*6*, *12*)

	Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
	DB 5	***DB WAX***							
			4-methylcyclopent-2-en-1-one	MS					(*6*)
			cyclopent-2-en-1-one	MS					(*6*)
	1093	1388	nonan-2-one	MS, RI		rue odor		5 µg/Kg	(*6*, *12*)
			cyclohex-2-en-1-one	MS					(*6*)
	1359		β-damascenone	MS, RI, GCO	LLB, LLC	fruity, woody, winey, berry	12800	0.0007 µg/Kg	(*6*, *13*)
			3-methyl-2-butanone	MS, RI	LLB, LLC				(*6*)
Phenols									
	1086	1872	guaiacol	MS, RI, GCO, ST	LLB, LLC	smoky, phenolic	3200	3 µg/Kg	(*6*, *13*, *21*)
			cresol	MS, RI					(*6*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***^{a}	***Threshold***	***References***
DB 5	***DB WAX***							
1074	2077	p-cresol	MS, RI	LLC	caracteristic phenol		55 µg/Kg	(*12*, *13*)
1285	2054	4-ethylguaiacol	MS, RI, GCO	LLB, LLC	smoky, phenolic	200	25 µg/Kg	(*6*, *13*, *21*)
1351	2175	eugenol	MS, RI, GCO, ST	LLB, LLC	spicy, clove	400	6 µg/Kg	(*6*, *13*, *21*)
1169	2170	3-ethylphenol	MS		musty			(*6*)
1410	2598	vanillin	MS, RI, GCO		sweet, creamy, vanilla, spicy	12800	0.1 mg/Kg	(*6*, *21*)
		syringic aldehyde	MS		alcoholic odor,weak sweet,slightly smoky,cinnamic,vanilla,medicinal nuance		1 mg/Kg	(*6*, *12*)
		coniferyl aldehyde	MS					(*6*)
1190	1890	4-methylguaiacol	GCO, MS	LLB, LLC	sweet, smoky, burnt wood	200	90 µg/Kg	(*6*, *13*, *21*)
1178	2195	4-ethyl phenol	MS	LLB, LLC	stable-like, horse		42 µg/Kg	(*13*, *21*)

	Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
	DB 5	***DB WAX***							
			diethyl phenol (3,4-diethylphenol/2,5-diethylphenol)	MS	LLB, LLC, SDE				(*13*)
	1299		carvacrol	MS, RI, ST	LLB, LLC, SDE	herbal phenolic		2.29 mg/Kg	(*12*, *13*)
Pyrazines									
	911	1320	2,5-dimethylpyrazine	MS, RI		musty,potato,cocoa and nutty		80 µg/Kg	(*6*, *12*)
	913	1308	2,6-dimethylpyrazine	MS, RI		nutty,coffee,cocoa		400 µg/Kg	(*6*, *12*)
	1419		2-ethyl-5-methylpyrazine	MS, RI		nutty,roasted,grassy		16 µg/Kg	(*6*, *12*)
	1001	1400	2-ethyl-3-methylpyrazine	MS, RI		strong raw-potato,roasted earthy		2 mg/Kg	(*6*, *12*)
	999		trimethylpyrazine	MS, RI		baked potato or roasted nut aroma		400 µg/Kg	(*6*, *12*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

	Retention Index		*Compound*	*Identification*	*Extraction*	*Odor descriptor*	*Max dilution*[a]	*Threshold*	*References*
	DB 5	*DB WAX*							
Sulfur compounds									
	738	824	dimethyl disulfide	MS, RI		intense onion odor		0.16 µg/Kg	(*6*, *12*)
	950	1377	dimethyl trisulfide	MS, RI, GCO		powerful,penetrating,reminiscent of fresh	100	2 mg/Kg	(*6*, *12*)
		1512	4-methyl-5-vinylthiazole	MS, RI		cocoa odor		20 mg/Kg	(*6*, *12*)
Terpenoids									
	1018	1186	1,4-cineole	MS, RI		cooling,minty menthol and herbal		40 mg/Kg	(*6*, *12*)
			linalyl ethyl ether	MS					(*6*)

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***[a]	***Threshold***	***References***
DB 5	***DB WAX***							
1074	1423	cis-linalool oxide	MS, RI	LLB, LLC, SDE	powerful,sweet, woody,penetrating odor with floral			(*6*, *12*, *13*)
1088	1453	trans-linalool oxide	MS, RI	LLB, LLC, SDE	sweet, floral, creamy, leafy			(*6*, *13*)
		geranyl ethyl ether	MS		diffusive ethereal fruity green			(*6*, *12*)
		p-cymen-8-yl ethyl ether	MS					(*6*)
1098	1551	linalool	MS, RI, GCO,ST	LLB, LLC, SDE	floral, sweet	800	4 µg/Kg	(*6*, *12*, *13*)
1178	1616	terpinen-4-ol	MS, RI, ST	LLB, LLC, SDE	citrus,tropical fruity		30 mg/Kg	(*6*, *12*, *13*)
		p-menth-1-en-9-al	MS	LLB				(*6*, *13*)
1354	1663	citronellyl acetate	MS, RI		fresh,fruity reminiscent of rose and a pungent		1 mg/Kg	(*6*, *12*)
		γ-terpineol	MS, RI					(*6*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

Retention Index		*Compound*	*Identification*	*Extraction*	*Odor descriptor*	*Max dilution*^{a}	*Threshold*	*References*
DB 5	***DB WAX***							
1233	1771	citronellol	MS, RI, GCO		sweet, floral, citrus fruits	25	11 µg/Kg	(*6*, *21*)
1228	1753	nerol	MS, RI	LLB, LLC	rose odor,slightly citrus,terpy and floral,reminiscent of linalool oxide and fruity nuance		680 µg/Kg	(*6*, *13*, *21*)
		nerolidyl ethyl ether	MS					(*6*)
1240	1850	geraniol	MS, RI		rose		4 µg/Kg	(*6*, *21*)
		trans-geraniol	MS, RI	LLB, LLC, SDE				(*13*)
1196	1887	p-cymen-8-ol	MS, RI		floral, sweet, citrusy		20 mg/Kg	(*6*, *12*)
		p-cymen-9-ol	MS					(*6*)
1565	2010	cis-nerolidol	MS, RI, ST	LLB, LLC, SDE	waxy, rose, apple, green, citrus			(*6*, *13*)
		6(E)-dihydrofarnesyl acetate	MS, GCO		fruity, woody, sweet	100		(*6*)

Retention Index		*Compound*	*Identification*	*Extraction*	*Odor descriptor*	*Max dilution*[a]	*Threshold*	*References*
DB 5	*DB WAX*							
1290		thymol	MS, RI, ST, GCO	LLB, LLC, SDE	warm, spicy, curry powder	1600	86 µg/Kg	(*6*, *13*)
		γ-bisabolol	MS, RI					(*6*)
		γ-farnesyl acetate	MS					(*6*)
		6(E)-dihydrofarnesol	MS					(*6*)
		trans,trans-γ-farnesol	MS, RI					(*6*)
1097	1586	myrcenol	MS, RI	LLB, LLC	fresh floral lavender citrus			(*6*, *13*)
1144		cis-β-terpineol	MS, RI	LLB, LLC	woody-earthy odor			(*12*, *13*)
1189	1711	α-terpineol	MS, RI, ST	LLB, LLC, SDE	lilac odor,pine,woody and resinous with a slightly cooling lemon and lime nuance		280 µg/Kg	(*12*, *13*)
		β-citronellol	MS, RI, ST	LLB, LLC, SDE				(*13*)

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Table I. (Continued). Compilation of flavor compounds found in tequila

	Retention Index		*Compound*	*Identification*	*Extraction*	*Odor descriptor*	*Max dilution*^{a}	*Threshold*	*References*
	DB 5	*DB WAX*							
			nerolidol oxide	MS	LLB, LLC				(*13*)
			α-bisabolool	MS	LLC				(*13*)
			cis-farnesol	MS	LLB				(*13*)
			trans-farnesol	MS	LLB, LLC, SDE				(*13*)
Miscellaneous compounds									
			prenyl ethyl ether	MS		fruity			(*6*, *12*)
			2,6,6-trimethyl-2-vinyltetrahydropyran	MS, RI		sweet, floral, citrus with woody, cooling, minty and camphoreous nuances			(*6*)

Retention Index		***Compound***	***Identification***	***Extraction***	***Odor descriptor***	***Max dilution***^{a}	***Threshold***	***References***
DB 5	***DB WAX***							
711	1291	acetoin	MS, RI, GCO		butter	400	10 mg/Kg	(*6*, *12*)
	1364	3-ethoxypropan-1-ol	MS					(*6*)
		3-methylbutyl phenylethyl ether	MS					(*6*)
		ethyl butyl ether	MS	LLB, LLC				(*13*)
		1,2,3,5-tetramethyl benzene	MS	LLB, LLC				(*13*)
		3-methyl-2-pentene	MS	LLB				(*13*)
	1538	2-methyl-tetrahydrothiophen-3-one	MS	LLB, LLC	pungent,alliaceous,coffee with a gasoline nuance		5 mg/Kg	(*12*, *13*)

[a] Maximum dilution found by aroma extract dilution analysis MS = Mass Spectrometry, RI = Kovats Retention Index, ST = Pure chemical standard, GCO = Gas Chormatography Olfactometry, LLB = Liquid-Liquid Batch extraction, LLC = Liquid-Liquid Continuous extraction, SDE = Simultaneous Distillation Extraction, SPME = Solid Phase Microextraction

Most Important Flavor Compounds

On the basis of their high dilution factor determined by a study using Aroma Extract Dilution Analysis, five compounds were selected as the most powerful odorants in gold tequila (*6*): isovaleraldehyde, isoamyl alcohol, β-damascenone, 2-phenylethanol and vainillin, which have cocoa, fusel, woody, floral and vanilla respectively. However, efforts at reconstituting tequila flavor by mixing these and other compounds found in the same study were unsuccessful, indicating that many key odorants have not yet been identified, and that there is a synergism between minor components. Moreover, only one type from one brand of tequila was analyzed in the study. There are five main types of tequila and hundreds of brands, many of them with highly variable processes, making chemical characterization of tequila flavor a great challenge.

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Chapter 16

Characteristic Aroma Compounds of Chinese Dry Rice Wine by Gas Chromatography–Olfactometry and Gas Chromatography–Mass Spectrometry

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Aroma compounds in Chinese rice wines were studied by gas chromatography–olfactometry (GC–O) and quantitative analysis. 57 aroma compounds were identified by GC–O followed by gas chromatography–mass spectrometry (GC–MS), among which 2-methylbutanol, 3-methylbutanol, butanoic acid, 3-methylbutanoic acid, 2-phenylethanol, phenol, 4-vinylguaiacol, furfural, and γ-nonalactone were identified with the highest aroma intensities. The quantitative analysis results shown 23 out of the quantified compounds could be found at concentrations higher than their corresponding odor thresholds in Chinese rice wines. On the basis of odor activity values (OAVs), the most potent odorants were dimethyl trisulfide. Other components, such as ethyl octanoate, ethyl butanoate, phenylacetaldehyde, ethyl hexanoate, 3-(methylthio) propanol, 2-phenylethanol, γ-nonalactone, and ethyl 2-methylpropanoate were also determined to be powerful odorants in Chinese rice wines.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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Introduction

Chinese rice wine is a traditional Chinese alcoholic beverage with more than 4,000 years of history. Due to its unique aroma, subtle flavor, and low alcoholic level, Chinese rice wine is widely consumed by Chinese all over the country. Compared with sake, Chinese rice wine has the characteristics of “yellow”, “sweet aroma”, and “abundant nutrition” (*1*, *2*). According to the total sugar content, Chinese rice wines could be sorted to four types: dry ($\leq$ 15.0 g/L), semi-dry (15.1–40.0 g/L), semi-sweet (40.1–100 g/L), and sweet styles ($\geq$ 100 g/L). Typically, alcoholic strength of dry type rice is more than 8% by volume, residue of no-sugar $\geq$ 20 g/L, total acids (as acetic acid) 3.5–7.0 g/L, amino nitrogen $\geq$ 0.5 g/L, pH 3.4–4.5, and β-phenylethanol $\geq$ 60 mg/L (Chinese National Standard GB/T 13662).

Chinese rice wine is mainly produced in the southern of China, such as Zhejiang province and Shanghai city, which are the most famous producing regions of Chinese rice wines. The traditional Chinese rice wine is produced by simultaneous saccharification and fermentation in the fermenter (Scheme 1). In this process, raw material of cereals (rice or/and sticky rice) is soaked in cold water overnight, cooked with steam, and wheat qu and yeast use as saccharifying and fermenting agents, respectively (*3*, *4*). After rice or/and sticky rice is cooked, 150% (w/w) water, approximately 10% (w/w) wheat qu, and defined amount yeast is added. And then, the saccharifying and fermentation is carried out in the semi-solid state in the ceramic vat (a kind of fermenter, now using closed stainless steel vessel) during 3–5 days. As soon as main fermentation finished, the fermented mash is transferred to stainless steel tank for postfermentation. The postfermentation last 15–30 days. The fresh rice wine is maturated in a sealed pottery jar at ambiance temperature for 1 to 3 years. The aged rice wine is generally blended to yield an ethanol content of 14−17% (by volume) for constant quality in the finished product (*4*).

The investigations on the volatile components of Chinese rice wine have been performed for a few years. More than 50 volatile and semi-volatile compounds were detected by liquid–liquid extraction (LLE), static headspace, and direct-injection gas chromatography (*1*). In 2008, Luo and co-workers (*3*) first employed headspace solid phase microextraction (HS–SPME) followed by gas chromatography–mass spectrometry (GC–MS) for analyzing the volatile and semi-volatile trace compounds of Chinese rice wines. A total of 97 volatile and semi-volatile compounds were detected and identified in several typical Chinese rice wines, including alcohols, acids, esters, aldehydes and ketones, aromatic compounds, lactones, phenols, sulfides, furans, and nitrogen-containing compounds. Up to now, no studies have published on aroma compounds in Chinese rice wine. The present work was intended for identifying the aroma compounds in typical Chinese rice wine by gas chromatography–olfactometry (GC–O), and for determining the concentrations of volatile compounds so as to find important aroma compounds.

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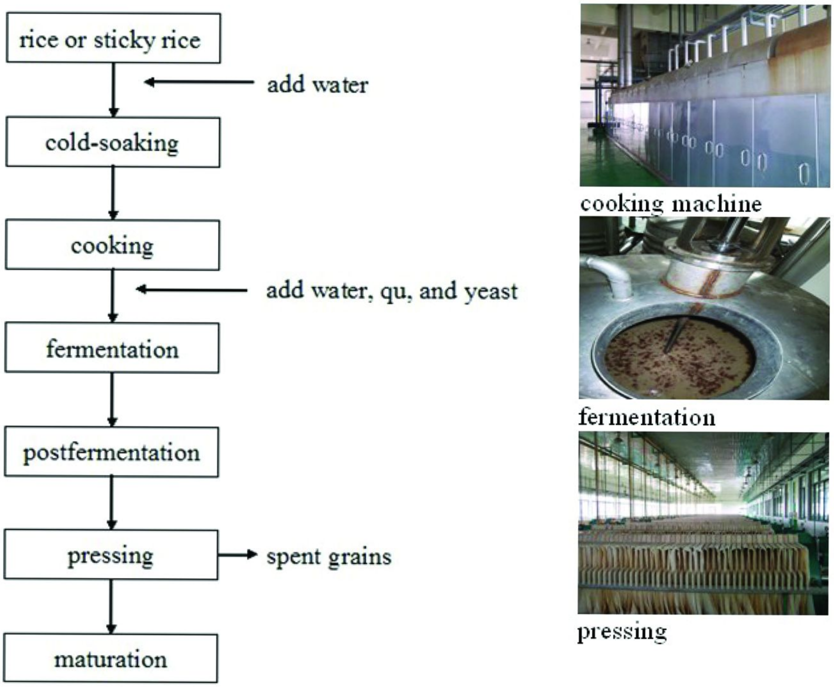
rice or sticky rice
add water
cold-soaking
cooking
add water, qu, and yeast
fermentation
postfermentation
pressing
spent grains
maturation
cooking machine
fermentation
pressing

Scheme 1. Producing process flowchart of Chinese rice wine

Materials and Methods

Chemicals

2-Octanol (96%), 3-methylbutanoic acid (98%), 2-phenylethanol (99%) and 2-phenylethyl acetate (99%) were from Fluka, Inc (Shanghai, China). 2-Methylpropanoic acid (99%) was from Alfa Aesar, Inc (Beijing, China). Butanoic acid (98%), pentanoic acid (98%) and octanoic acid (98%) were from Ciyun Chemical Company (Wujiang, Jiangsu, China). Hexanal was from Peking University Zoteq Co., Ltd (Beijing, China). Others were from Sigma–Aldrich China Co. (Shanghai, China).

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Samples

Commercial Chinese Rice Wines

Two samples (12–14% ethanol by volume) of commercially available brands of Chinese rice wine, which typically manufactured by two Chinese rice wine company located in Shaoxing and Shanghai of China, respectively, were gifted by these two companies. The sample from Shanghai was labeled as JF12 and the other sample was labeled as GY30. All samples belong to the dry type rice wine.

Synthetic Rice Wine

Synthetic rice wine was produced by mixing 2.5 g/L lactic acid and 12% (by volume) ethanol-water solution. The pH was adjusted to 4 by addition of 6 mol/L NaOH.

Aroma Extraction and Fractionation

Aroma Extraction

A total of 200 mL of each Chinese rice wine sample was diluted with deionized water (boiled for 5 min and then cooled to room temperature) by the volume ratio of 1:1. The diluted rice wine sample was saturated with analytical-grade sodium chloride and extracted 3 times with 200 mL aliquots of freshly distilled diethyl ether : pentane (1:1 by volume) in a separating funnel. All extracts were combined and slowly concentrated to 100 mL under a gentle stream of nitrogen. This was labeled as “extract 1. ”

Acidic/Water-Soluble Fraction

To facilitate GC–O and GC–MS analysis, the aroma extract of rice wine was separated into acidic/water-soluble, neutral, and basic fractions, using a modified method of Fan and Qian (*5*). A total of 50 mL of deionized water was added to “extract 1”. The aqueous phase was adjusted to pH 10 with sodium bicarbonate solution (10%, w/v), and then separated in a separating funnel and retained. The organic phase was washed 2 times with 10 mL of deodorized water. The washings were combined with the aqueous phase. The organic phase was labeled “extract 2. ”

The combined aqueous phase was further adjusted to pH 2 with 2 N H_2SO_4, saturated with NaCl, and then extracted 3 times with 30 mL freshly distilled diethyl ether : pentane (1:1 by volume). The extracts were combined and dried with 5 g of anhydrous sodium sulfate overnight. The dried solution was filtered and then slowly concentrated to a final volume of 200 μL under a gentle stream of nitrogen. This concentrate was labeled as the “acidic/water-soluble fraction” for further GC–O analysis.

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Basic Fraction

A total of 50 mL of deodorized water was added to “extract 2”. The aqueous phase was adjusted to pH 2 with 2 N H_2SO_4, saturated with NaCl, and then separated in a separating funnel. The organic phase was labeled “extract 3” and saved. The aqueous phase was then adjusted to pH 10 with sodium bicarbonate solution (10%, w/v) and then extracted 3 times with 30 mL of freshly distilled diethyl ether : pentane (1:1 by volume). The organic phase was combined and dried with 5 g anhydrous sodium sulfate overnight. The extract was filtered and slowly concentrated to 200 μL under a gentle stream of nitrogen. This extract was labeled as the “basic fraction”.

Neutral Fraction

The “extract 3” was dried with 5 g anhydrous sodium sulfate overnight. The extract was filtered and slowly concentrated to 200 μL under a gentle stream of nitrogen. This extract was labeled as the “neutral fraction”.

GC–O Analysis

GC–O analysis was performed on a Agilent 6890 GC equipped with an olfactometer (ODP2, Gerstel Inc., Germany). The column carrier gas was nitrogen, at a constant flow rate of 2 mL/min. Half of the column flow was directed to the detector, while the other half was directed to the olfactometer. The samples were analyzed on a DB-Wax column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA) and a DB-5 column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA). Each concentrated fraction (1 μL) was injected with a splitless mode. The oven temperature was held at 50 °C for 2 min, then raised to 230 °C at a rate of 6 °C/min, and held at 230 °C for 15 min. Injector and sniffing port temperature was 250 °C.

Three panelists (1 female and 2 males) were selected for the GC–O study. They had both been trained more than 6 months for GC–O analysis. They responded to the aroma intensity of the stimulus by using a 6-point scale ranging from 0 to 5; ‘0’ was none, ‘3’ was moderate, while ‘5’ was extreme. The retention time, intensity value, and aroma descriptor were recorded. Each fraction was replicated 3 times by each panelist. The Osme values for aroma intensity were averaged for the nine analyses (3 panelists, 3 times). When a panelist could not detect a aroma compound, the intensity was considered as zero in the averaging process.

GC–MS Analysis

The analyses were carried out on a Agilent 6890 GC equipped with a Agilent 5975 mass selective detector (MSD). The samples were analyzed on a DB-Wax column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA) and a DB-5 column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W

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Scientific, Folsom, CA). The GC condition was identical to the GC–O analysis described above. The carrier gas was helium with a flow-rate of 2 mL/min. A Agilent 5975 MSD was used for identification of unknown compounds. The electron impact energy was 70 eV, and the ion source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. Electron impact (EI) mass spectra were recorded in the 35–350 amu range.

Retention Indices (RI)

Retention indices (RI) were calculated in accordance with a modified Kovats method (*6*). A standard mixture of paraffin homologues C5–C25 was prepared. The sample and the hydrocarbon standard mixture were co-injected into the GC and the retention times were used to calculate retention indices.

Quantitative Method

HS–SPME and GC–MS Analysis

Quantitative analysis of the aroma compounds was carried out using the method proposed and validated by Luo and co-workers (*3*). An automatic headspace sampling system (MultiPurposeSample MPS 2 with a SPME adapter, from GERSTEL Inc., Baltimore, MD) with a 50/30 μm divinylbenzene/carboxen /poly(dimethylsiloxane) (DVB/CAR/PDMS) fiber (2 cm, Supelco, Inc., Bellefonte, PA) was used for extraction of volatile compounds. For headspace sampling, each rice wine sample was diluted with freshly redistilled–deionized water to a final concentration of 6% (by volume) of ethanol (*3*). A total of 8 mL of diluted sample was put into a 20 mL autosampler vial and spiked with 5 μL of internal standards (ISs, 2-octanol and geranyl acetate) solution at 1 mg/L in absolute ethanol. The diluted sample was saturated with sodium chloride. And the vial was capped with a Teflon septum and an aluminium cap. This sample was equilibrated at 50 °C for 5 min and extracted for 45 min at the same temperature under stirring (250 rpm, on for 20 s, off for 0 s). After extraction, the fiber was automatically inserted into the injection port of GC (250 °C) for 5 min to desorb the analytes. All analyses were repeated in triplicate. The oven and injector temperatures were identical to GC–O analysis described above on a DB-Wax column. Selected ion monitoring (SIM) mode was applied in the quantitative analysis.

Calibration of Standard Curves

Four sets of synthetic rice wine containing difference concentrations of standard compounds (1st group of all alcohols, 2nd group of all acids, 3rd group of all esters, and 4th group of other compounds, Table 2) was diluted to 6% (by ethanol volume), and spiked with ISs standard solution, respectively.

These diluted standard samples were placed into 20 mL autosampler vial. The HS–SPME conditions and GC–MS conditions were set as described previously. The standard curves for individual compounds were built up by plotting the response ratio of target compound and ISs against the concentration ratio. According to the retention time, the internal standard which was closest to the target component was selected.

Calculation of Recovery

Known amounts of standard aroma compounds were evaluated in synthetic liquor, as well as in the samples. The concentrations of these aroma compounds in rice wines before and after addition of standard aroma compounds were quantified as described previously.

$$\mathrm{Re}(\%) = \frac{(c_x - c_0) \times v_0}{A} \times 100\%$$

Re is the recovery; C_x is the concentration of certain aroma compounds detected after addition A μg, μg/L; C_0 is the concentration detected before addition, μg/L; v_0 is the volum of sample, L; and A is the amount added to the sample, μg.

Results and Discussion

Identification of Aroma Compounds

To facilitate the identification of aromas, the extract of rice wine sample was separated into three fractions: acidic/water-soluble, basic, and neutral. GC–O and GC–MS were performed on each fractionation. A total of 57 aroma compounds were identified on DB-Wax and DB-5 columns in these two samples (Table 1), including 9 alcohols, 9 esters, 8 fatty acids, 10 aromatic compounds, 7 phenolic derivates, 5 furans, 1 lactone, 2 sulfur-containing compounds, 4 nitrogen-containing compounds, 1 aldehyde, and 1 ketone. Among these, 5 aroma compounds, unknowns, were detected by GC–O but could not be identified by GC–MS.

Like other alcoholic beverages, alcohols were the main volatile aroma compounds in Chinese rice wines. On the basis of the Osme values, the potentially important alcohols were 2-methylbutanol and 3-methylbutanol, and imparted alcoholic and nail polish notes, respectively. 1-Propanol and 2-methylpropanol could also be important aroma compounds because they had medium Osme values. 1-Propanol had fruity and alcoholic aromas, whereas 2-methylpropanol contributed wine and solvent notes. Higher alcohols could be formed during the fermentation, under aerobic conditions from sugar and anaerobic conditions from amino acids (*3*, *7*). Since the raw materials (rice and wheat) are rich sources

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of amino acids, amino acids could be converted to higher alcohols by yeast via Ehrlic metabolic pathway. A small amount of higher alcohols could also be made by yeast through reduction of corresponding aldehydes (*8*, *9*).

Esters of fatty acids especially ethyl esters in both rice wines had medium Osme values. The aroma intensities of ethyl acetate, ethyl propanoate, ethyl butanoate, and ethyl hexanoate were strong and contributed pineapple, floral, and fruity notes to Chinese rice wines. The aroma intensity of diethyl butanedioate in JF12 rice wine, which had fruity and sweet aromas, was much higher than in GY30. 3-Methylbutyl acetate, which gave fruity aroma, was identified with weak aroma intensities in both JF12 and GY30 rice wine. The aroma intensities of other esters were weak, and which gave fruity, sweet, and banana aromas. Esters were mostly formed through esterification of alcohols with fatty acids during fermentation and aging process. Ester formation can be influenced by many factors such as fermentation temperature, oxygen availability, and fermentation strains (*10*).

A total of 8 fatty acids were identified in these two Chinese rice wines by GC–O and GC–MS. On the basis of Osme values, the potentially important fatty acids in both samples were butanoic acid and 3-methylbutanoic acid (Osme values > 4.33) and contributed to rancid, acidic, and cheesy notes. Acetic, propanoic acid, and 2-methylpropanoic acid were found in both JF12 and GY30 rice wine, but they had lower Osme values. The aroma intensities of fatty acids in GY30 rice wine were stronger than in JF 12 rice wine. Most of the fatty acids in Chinese rice wines were produced by yeast metabolism (*3*). And some would be from raw material and wheat qu.

There were 10 aroma-active aromatic compounds identified in both rice wines. The aroma intensity of 2-phenylethanol, which could be produced by *Saccharomyces cerevisiae* (*11*), was strongest among these all aromatic compounds, and it contributed to honey and rose aromas. Some other aromatic compounds, including benzaldehyde, phenylacetaldehyde, and 1-phenyl-1-propanone (propiophenone, tentatively identified), had medium aromas (Osme = 3–3.67). They contributed to fruity, berry, floral, rose, and pungent aromas, and could be important to the aroma of Chinese rice wine. Acetophenone, ethyl benzoate, ethyl 2-phenylacetate, Z-2-phenyl-2-butenal, and ethyl 3-phenylpropanoate had low aroma intensities (Osme values = 1–2.67). Acetophenone gave a musty, almond, and glue aromas, and ethyl benzoate gave fruity aroma, whereas ethyl 2-phenylacetate had fruity and sweet aromas. 2-Phenylethyl acetate was only identified in GY30 rice wine and gave rosy and floral aromas. Aromatic compounds were could mainly formed through aromatic amino acids metabolism (*12*).

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Table 1. Aroma Compounds in JF12 and GY30 Detected by GC–O

RI_{Wax}	*$RI_{DB\text{-}5}$*	*aroma compound*	*descriptor*	*fraction* [a]	*basic of identification* [b]	*Osme value*	
						JF12	*GY30*
alcohols							
1035	530	1-propanol	fruity, alcoholic	A/W	MS, RI, aroma	2.67	2.17
1087	618	2-methylpropanol	wine, solvent	A/W	MS, RI, aroma	2.67	3.50
1137	643	1-butanol	rancid	A/W	MS, RI, aroma	2.17	2.33
1195	753	2-methylbutanol	alcoholic	A/W	MS, RI, aroma	3.50	4.00
1201	783	3-methylbutanol	nail polish, rancid	A/W	MS, RI, aroma	3.50	4.00
1268	798	1-pentanol	fruity, balsamic	A/W	MS, RI, aroma	2.00	2.50
1341	888	1-hexanol	floral, green	A/W	MS, RI, aroma	ND [c]	1.67
1448	986	1-octen-3-ol	mushroom	A/W	RI, aroma	2.50	ND
1443	984	1-heptanol	alcoholic, fruity	A/W	RI, aroma	2.00	2.00
esters							
892	584	ethyl acetate	pineapple	N	MS, RI, aroma	3.00	2.67
953	705	ethyl propanoate	fruity, banana	N	MS, RI, aroma	3.00	2.00
961	754	ethyl 2-methylpropanoate	fruity, sweet	N	MS, RI, aroma	2.17	1.17
1031	800	ethyl butanoate	pineapple	N	MS, RI, aroma	3.00	3.00
1102	875	3-methylbutyl acetate	fruity	N	MS, RI, aroma	2.00	2.83

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Table 1. (Continued). Aroma Compounds in JF12 and GY30 Detected by GC–O

RI_{Wax}	$RI_{DB\text{-}5}$	*aroma compound*	*descriptor*	*fraction* [a]	*basic of identification* [b]	*Osme value*	
						JF12	*GY30*
1128	900	ethyl pentanoate	apple	N	MS, RI, aroma	2.67	1.17
1235	1010	ethyl hexanoate	fruity, floral, sweet	N	MS, RI, aroma	3.50	3.17
1409	1196	ethyl octanoate	fruity	N	RI, aroma	1.50	1.67
1655	1176	diethyl butanedioate	fruity, sweet	N	MS, RI, aroma	3.17	1.50
fatty acids							
1424	582	acetic acid	acidic, vinegar	A/W	MS, RI, aroma	3.50	4.33
1555	789	2-methylpropanoic acid	rancid, acidic	A/W	MS, RI, aroma	3.67	3.33
1602	802	butanoic acid	rancid, cheesy	A/W	MS, RI, aroma	4.33	4.50
1655	877	3-methylbutanoic acid	rancid, acidic	A/W	MS, RI, aroma	4.33	4.67
1727	911	pentanoic acid	sweat, rancid	A/W	MS, RI, aroma	2.17	2.67
1846	1019	hexanoic acid	sweat, cheesy	A/W	MS, RI, aroma	2.17	2.33
1955	1103	heptanoic acid	sweat	A/W	MS, RI, aroma	0.83	2.33
2060	1171	octanoic acid	sweat, cheesy	A/W	MS, RI, aroma	2.17	0.67
aromatic compounds							
1501	963	benzaldehyde	fruity, berry	N	MS, RI, aroma	3.00	3.67
1620	1047	phenylacetaldehyde	floral, rose	N	MS, RI, aroma	3.00	3.33

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RI_{Wax}	*RI_{DB-5}*	*aroma compound*	*descriptor*	*fraction* [a]	*basic of identification* [b]	*Osme value*	
						JF12	*GY30*
1625	1035	acetophenone	sweet, fruity, floral	N	MS, RI, aroma	2.00	2.00
1640	1175	ethyl benzoate	fruity	N	MS, RI, aroma	1.50	2.00
1694		1-phenyl-1-propanone [d]	pungent, floral	N	MS, aroma	3.33	3.17
1768	1247	ethyl 2-phenylacetate	rosy, honey	N	MS, RI, aroma	1.67	2.17
1801	1260	2-phenylethyl acetate	rosy, floral	N	MS, RI, aroma	ND	2.50
1873	1353	ethyl 3-phenylpropanoate	rose, floral	A/W	MS, RI, aroma	3.00	2.00
1906	1116	2-phenylethanol	honey, rose	A/W	MS, RI, aroma	4.00	3.83
1916	1276	Z-2-phenyl-2-butenal	cocoa, sweet, rum	N	MS, RI, aroma	1.17	2.67
phenolic derivates							
1858	1090	guaiacol	spicy, clove, animal	A/W	MS, RI, aroma	1.50	2.67
1952	1195	4-methylguaiacol	smoke	A/W	MS, RI, aroma	2.33	ND
2007	987	phenol	phenol, medicinal	A/W	MS, RI, aroma	3.17	3.17
2080	1082	4-methylphenol	animal, phenol	A/W	MS, RI, aroma	2.67	ND
2185	1181	4-ethylphenol	smoky	A/W	MS, RI, aroma	2.50	ND
2200	1323	4-vinylguaiacol	spicy, clove	A/W	MS, RI, aroma	3.67	3.67
2208	1345	2,6-dimethoxyphenol [d]	smoke	A/W	RI, aroma	1.67	ND
furans							

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Table 1. (Continued). Aroma Compounds in JF12 and GY30 Detected by GC–O

RI_{Wax}	*$RI_{DB\text{-}5}$*	*aroma compound*	*descriptor*	*fraction* [a]	*basic of identification* [b]	*Osme value*	
						JF12	*GY30*
1456	831	furfural	almond, sweet	N	MS, RI, aroma	4.50	4.50
1489	917	2-acetylfuran	sweet, caramel	N	MS, RI, aroma	2.50	2.33
1555	967	5-methyl-2-furfural	green, roasted	N	MS, RI, aroma	ND	1.50
1603	1058	ethyl 2-furoate	balsamic	N	MS, RI, aroma	1.83	2.00
1647	854	2-furanmethanol	burnt sugar	A/W	MS, RI, aroma	1.50	1.33
lactones							
2018	1363	γ-nonalactone	coconut, peach	N	MS, RI, aroma	4.83	4.83
aldehydes and ketones							
1073	797	hexanal	green, grass, apple	N	MS, RI, aroma	1.67	0.83
1300	980	1-octen-3-one [d]	mushroom, earthy	N	RI, aroma	2.17	2.17
sulfur-containing compounds							
1360	976	dimethyl trisulfide	sulfur, rotten cabbage	N	Aroma, RI	2.67	2.50
1702	978	3-(methylthio)propanol	cooked vegetable	A/W	MS, RI, aroma	2.67	3.00
nitrogen-containing compounds							
1315	915	2,5-dimethylpyrazine	baked, nutty	B	MS, RI, aroma	2.67	2.67
1330	910	2,6-dimethylpyrazine	nutty	B	MS, RI, aroma	2.67	2.67
1430	1089	2,5-dimethyl-3-ethylpyrazine [d]	roasted, baked	B	RI, aroma	2.67	1.67

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RI_{Wax}	*$RI_{DB\text{-}5}$*	*aroma compound*	*descriptor*	*fraction* [a]	*basic of identification* [b]	*Osme value*	
						JF12	*GY30*
1972	1024	2-acetylpyrrole	herbal, medicine	B	MS, RI, aroma	1.17	2.33
Unknowns							
1314		unknown	cooked rice	B		2.33	2.67
1417		unknown	fatty, oily	N		2.50	ND
1762		unknown	acid, sour	A/W		2.00	ND
1783		unknown	sweet, flower	N		ND	2.17
1949		unknown	caramel	N		1.50	2.50

[a] A/W, acidic/water-soluble fraction; N, neutral fraction; B, basic fraction. [b] MS, compounds were identified by MS spectra; aroma, compounds were identified by the aroma descriptors; RI, compounds were identified by a comparison to the pure standard. [c] ND: not detected by GC–O. [d] tentatively identified.

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Table 2. Calibration Data of Aroma compound Standards and Their Recovery in Chinese Rice wine (n = 3)

aroma compounds	*SIM*	*slope*	*intercept*	*R^2*	*n*	*linear rage (μg/L)*		*LOD (μg/L)*	*ISs*[a]	*recovery (%)*		
						min	*max*			*SYN* [c]	*JF12*	*GY30*
alcohols												
1-propanol	31	0.0016	–0.0450	0.9981	9	732.48	151300.00	606.46	2–O	118	87	112
2-methylpropanol	43	0.0023	0.0270	0.9941	8	525.86	154250.00	158.55	2–O	91	104	75
1-butanol	56	0.0019	–0.0130	0.9944	10	268.15	17161.84	90.39	2–O	102	74	86
2-methylbutanol	57	0.0099	–0.2797	0.9938	10	120.88	146900.00	5.38	2–O	91	104	127
3-methylbutanol	55	0.0048	0.0548	0.9918	10	195.52	155000.00	2.66	2–O	75	81	124
1-pentanol	42	0.0063	–0.0367	0.9953	9	130.39	16689.60	18.03	2–O	77	ND [b]	ND
1-hexanol	56	0.0482	–0.0344	0.9976	10	9.55	4890.95	3.46	2–O	90	95	111
1-octen-3-ol	57	0.5093	–0.0772	0.9996	8	2.46	5037.91	1.19	2–O	102	104	104
1-heptanol	70	0.1520	–0.0506	0.9999	9	1.69	3467.71	1.02	2–O	84	ND	75
esters												
ethyl acetate	43	0.0135	–0.4189	0.9973	9	203.56	156332.26	140.38	2–O	92	137	82
ethyl propanoate	57	0.0463	–0.0255	0.9977	9	9.10	2330.00	7.16	2–O	86	113	90
ethyl 2-methylpropanoate	71	0.0511	–0.0674	0.9967	9	12.86	3293.28	7.55	2–O	83	110	107
ethyl butanoate	71	0.0600	–0.2368	0.9981	10	14.04	14376.53	6.99	2–O	93	111	105
3-methylbutyl acetate	43	0.2470	0.0155	0.994	10	2.04	4184.46	0.53	2–O	105	92	81
ethyl pentanoate	88	0.1463	0.0237	0.9975	10	2.26	4627.50	1.63	2–O	105	100	ND

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aroma compounds	*SIM*	*slope*	*intercept*	*R^2*	*n*	*linear rage (μg/L)*		*LOD (μg/L)*	*ISs*[a]	*recovery (%)*		
						min	*max*			*SYN* [c]	*JF12*	*GY30*
ethyl hexanoate	88	0.6866	–0.3333	0.9934	10	2.72	2781.88	1.38	2–O	97	93	72
ethyl octanoate	88	1.9870	–2.1765	0.9907	9	2.51	2573.26	1.50	2–O	93	81	74
diethyl butanedioate	101	0.0481	0.0668	0.9964	10	8.19	8387.98	4.00	2–O	92	112	96
fatty acids												
acetic acid	43	0.0002	0.0644	0.9903	8	1480.59	189515.00	955.22	2–O	91	87	111
2-methylpropanoic acid	43	0.0230	–0.1115	0.9894	8	74.26	19010.00	39.88	2–O	41	31	40
butanoic acid	60	0.0058	0.0136	0.991		72.70	18610.00	38.42	2–O	39	ND	70
3-methylbutanoic acid	60	0.0046	–0.1086	0.9971	8	399.86	204730.00	234.07	2–O	60	43	18
pentanoic acid	60	0.0060	–0.0136	0.9962	8	122.42	15670.00	61.99	2–O	68	48	ND
hexanoic acid	60	0.0065	0.0158	0.9902	8	39.52	10117.50	18.49	GE	52	31	34
heptanoic acid	60	0.0226	0.0231	0.9958	8	18.76	1200.94	8.30	GE	58	ND	38
octanoic acid	60	0.0479	0.0167	0.9997	8	18.82	2409.38	7.43	GE	66	39	39
aromatic compounds												
benzaldehyde	106	0.3808	1.0689	0.9963	10	2.10	10081.06	1.50	2–O	103	66	65
phenylacetaldehyde	91	0.2897	–0.0298	0.9995	10	1.73	887.99	0.97	2–O	114	107	101
acetophenone	105	0.6711	0.1224	0.9977	10	0.48	985.82	0.04	2–O	93	90	60
ethyl benzoate	105	2.2572	–0.2144	0.9986	10	2.47	2526.55	0.92	2–O	98	85	71

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Table 2. (Continued). Calibration Data of Aroma compound Standards and Their Recovery in Chinese Rice wine (n = 3)

aroma compounds	*SIM*	*slope*	*intercept*	*R^2*	*n*	*linear rage (µg/L)*		*LOD (µg/L)*	*ISs^a*	*recovery (%)*		
						min	*max*			*SYN ^{c}*	*JF12*	*GY30*
ethyl 2-phenylacetate	91	1.5226	0.0862	0.9916	10	1.24	318.13	0.34	GE	96	80	85
2-phenylethyl acetate	104	0.9497	–0.0831	0.9924	10	1.63	417.75	0.27	GE	101	82	89
ethyl 3-phenylpropionate	104	1.5211	0.0031	0.9921	8	0.96	490.00	0.18	GE	102	ND	78
2-phenylethanol	91	0.0127	0.4279	0.9913	10	29.01	172050.00	9.09	GE	102	109	118
Z-2-phenyl-2-butenal	115	0.4839	0.0607	0.9722	9	1.57	1611.4958	0.81	GE	93	102	97
phenolic drivates												
guaiacol	109	0.0265	–0.0021	0.9935	8	7.64	244.57	7.25	GE	91	ND	82
4-methylguaiacol	138	0.0538	–0.0045	0.9997	7	5.47	700.28	4.99	GE	93	ND	ND
phenol	94	0.0317	–0.0016	0.9907	6	9.68	154.83	4.86	GE	95	ND	ND
4-methylphenol	107	0.0461	–0.0067	0.9981	9	5.64	722.48	3.24	GE	93	ND	Nd
4-ethylphenol	107	0.1121	–0.0022	0.9955	9	1.14	1171.38	0.96	GE	86	85	79
4-vinylguaiacol	150	0.0275	–0.0133	0.9917	8	13.69	3503.63	10.70	GE	111	ND	ND
furans												
furfural	96	0.0196	0.2795	0.9904	10	15.34	47137.50	14.73	2–O	98	133	112
2-acetylfuran	95	0.0345	0.0223	0.9977	9	22.85	2924.95	12.93	2–O	81	ND	ND
5-methyl-2-furfural	110	0.0520	–0.0577	0.9976	9	6.20	3174.86	3.63	2–O	88	55	64

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aroma compounds	*SIM*	*slope*	*intercept*	*R^2*	*n*	*linear rage (μg/L)*		*LOD (μg/L)*	*ISs*[a]	*recovery (%)*		
						min	*max*			*SYN* [c]	*JF12*	*GY30*
ethyl 2-furoate	95	0.2528	0.0017	0.9991	9	4.20	1074.44	3.82	2–O	94	ND	149
2-furanmethanol	98	0.0012	–0.0181	0.9938	6	45.01	5760.67	42.50	2–O	92	ND	ND
lactones												
γ-nonalactone	85	0.1247	0.0366	0.9947	10	2.22	1135.35	1.29	GE	98	75	90
aldehydes												
hexanal	44	0.2305	–0.0147	0.9994	10	1.47	1508.54	0.57	2–O	101	94	89
sulfur-containing compounds												
dimethyl trisulfide	126	0.5492	–2.3421	0.9943	9	40.47	5180.21	0.39	2–O	89	ND	ND
3-(methylthio)propanol	106	0.0005	0.0308	0.9905	10	104.49	20062.62	73.65	2–O	95	107	76
nitrogen-containing compounds												
2,5-dimethylpyrazine	108	0.0299	0.0039	0.9974	9	6.56	839.81	2.15	2–O	89	ND	80
2,6-dimethylpyrazine	108	0.0253	0.0067	0.9963	9	7.51	1922.29	6.42	2–O	89	ND	81
2-acetylpyrrole	94	0.0161	0.0012	0.9862	8	31.23	999.50	24.66	GE	89	ND	ND

[a] 2-O, 2-octanol; GE, geranyl acetate. [b] ND, not detected. [c] SYN, synthetic rice wine

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Total of 7 phenolic derivates were detected in these two Chinese rice wines. Only 3 phenolic derivates were identified in GY30 rice wine. 4-Vinylguaiacol and phenol had Osme values > 3 in both rice wines and contributed to strong clove, spicy, and smoky aromas. 4-Methylguaiacol, 4-methylphenol, 4-ethylphenol, and 2,6-dimethoxyphenol (tentatively identified) were only identified by GC–O in JF12 rice wine. These compounds contributed smoky, animal, and phenolic aromas. The Osme value of phenolic derivates in these two samples was obviously different. Phenolic derivates in Chinese rice wines would belong to the secondary plant constituents, mainly derived from lignin degradation (*13*).

Furans were also found to be important to both Chinese rice wine aroma profiles. There were 5 furans identified in both Chinese rice wines. Furfural had the strongest aroma intensity (Osme value = 4.50) among all furans and contributed to almond and sweet aromas. 2-furanmethanol had the medium aroma intensity (Osme value = 3.33–3.50). 2-Acetylfuran and ethyl 2-furoate were detected with weak aroma intensities in both samples. 5-Methylfufural was only detected in GY30 rice wine with very low aroma intensities. As a result of cooking process, furans were formed by thermal degradation and rearrangement of carbohydrate and protein in non-enzymic browning reactions (Maillard reaction) (*14*).

Two aroma active sulfur-containing compounds were identified in both Chinese rice wines. Dimethyl trisulfide and 3-(methylthio)propanol (methionol) were identified in both rice wines. The aroma intensities of these two compounds were more than 2.5. However, due to their very low aroma threshold, sulfur-containing compounds may be important for Chinese rice wine aroma. Sulfur-containing compounds probably came from the degradation of sulfur-containing amino acids (*15*).

Only 1 lactone (γ-nonalactone) was identified in both samples with strong aroma intensities (Osme value = 4.83). It mainly contributed to coconut and peach aromas, and could be mainly produced by bacteria (*16*). Four nitrogen-containing compounds were detected by GC–O in this study with very low aroma intensities. 2-Acetylpyrrole had herbal and medicine aromas, while others gave nutty and roasted aromas. Pyrazines could be less important, but they were important in Chinese liquors (*17*). Only 1 aldehyde and 1 unsaturated ketone were detected by GC–O and GC–MS in both JF12 and GY30 rice wine. Hexanal gave green, grass, and apple aromas. 1-Octen-3-one, tentatively identified, had mushroom and earthy aromas, and which identified in both rice wines.

Quantification Method of Aroma Compounds

HS–SPME and GC–MS had applied to analyze the volatile and semi-volatile compounds in Chinese rice wines (*3*). Therefore, this method was selected to quantitative analysis the aroma compounds identified by GC–O. Synthetic rice wine and JF12 were used for method validation, respectively. The limits of detection (LODs) were established for signal to noise ratios of 3. The quantitative ions, linearity data, limits of detection, recovery, and RSD of aroma compounds in synthetic rice wine and sample were summarized in Table 2. Eight-point calibrations were performed in this study.

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For most aroma compounds, linear responses were obtained with R^2 ranging from 0.9902 (hexanoic acid) to 0.9999 (1-heptanol), except 2-acetylpyrrole (R^2= 0.9894) and 2-methylpropanoic acid (R^2=0.9862), showing good linearity in the concentration range under consideration. The RSDs obtained varied from 0.38% (1-butanol) to 10.91% (ethyl benzoate). The RSDs of acetic, 2-methylpropanoic, and butanoic acid were higher than 20% in this study. The good repeatability of this method could be deduced from the low RSDs values except individual compounds. As shown in Table 2, the LODs of the most aroma compounds were from 0.04 μg/L (acetophenone) to 90.39 μg/L (1-butanol), except some compounds (such as acetic acid, 3-methylbutanoic acid, 1-propanol, etc.) with strong polarity had much higher LODs. The recoveries for most analytes ranged from 80.79% to 117.74%. Some compounds had low recoveries could be affected by the matrix effect (*18*) that causes the recovery to be far inferior to the levels expected. In total, these results indicated that the proposed method was suitable for the analysis of these analytes.

OAVs of Aroma Compounds in Chinese Rice Wines

The results of quantitative analysis (Table 3) showed that the three largest group were fatty acids, alcohols, and aromatic compounds, whereas the three highest concentrations of the aroma compounds quantified in two samples were 3-methylbutanol (122043.16–129286.17 μg/L), 2-phenylethanol (76160.94–133472.45 μg/L), and acetic acid (44722.55–265223.95 μg/L). The short chain fatty alcohols were the main alcohols in these Chinese rice wines, especially the concentrations of 3-methylbutanol were higher than 120000 μg/L in both samples. The concentrations of fatty acids in GY30 were much higher than in JF12 rice wine. Due to the different brewing technique, the concentration of acetic acid in GY30 was much higher than that in JF12 rice wine. According to the concentrations of these compounds in Chinese rice wines, the most abundant aromatic compound were 2-phenylethanol. Ethyl esters of fatty acids were the mainly esters in Chinese rice wines. Furfural and 3-(methylthio)propanol were the other two high concentration compounds in these two samples. Due to some compounds' low concentrations or their very polar in Chinese rice wines, these compounds identified by GC–O were not detected or less than the method detection limit.

Data in Table 3 indicated that the concentrations of 23 aroma compounds quantified in Chinese rice wines could be higher than their corresponding odor thresholds, among which, the OAVs of 15 and 19 aroma compounds quantified in JF12 and GY30 respectively were more than 1.

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Table 3. Quantitative Data and OAVs of Aroma Compounds in both JF12 and GY30 (n=3)

aroma compounds	*odor threshold (μg/L)*	*JF12*			*GY30*		
		concn (μg/L)	*RSD%*	*OAV*	*concn (μg/L)*	*RSD%*	*OAV*
alcohols							
1-propanol	306000 (*21*)	11312.40	1.97	< 1	30015.02	4.65	< 1
2-methylpropanol	40000 (*22*)	42709.37	0.11	1.1	33123.41	1.87	< 1
1-butanol	150000 (*23*)	1237.36	2.42	< 1	2758.82	0.71	< 1
2-methylbutanol		19931.98	8.99	−	23242.20	3.18	−
3-methylbutanol	30000 (*22*)	129286.17	0.42	4.3	122043.16	0.76	4.1
1-pentanol		< q.l. [a]	−	−	< q.l.	−	−
1-hexanol	8000 (*22*)	197.53	0.28	< 1	524.69	0.10	< 1
1-octen-3-ol	40 (*24*)	19.28	0.39	< 1	20.43	0.87	< 1
1-heptanol	3000 (*25*)	< q.l.	−	−	48.86	9.31	< 1
esters							
ethyl acetate	7500 (*22*)	31512.13	0.79	4.2	29302.84	0.83	3.9
ethyl propanoate	1800 (*26*)	403.89	0.83	< 1	304.94	0.42	< 1
ethyl 2-methylpropanoate	15 (*27*)	279.42	1.21	18.6	< q.l.	−	−
ethyl butanoate	20 (*22*)	636.49	0.59	31.8	444.57	0.56	22.2
3-methylbutyl acetate	30 (*22*)	65.69	2.55	2.2	24.88	9.33	< 1
ethyl pentanoate	10 (*28*)	34.72	1.74	3.5	< q.l.	−	−

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aroma compounds	*odor threshold (µg/L)*	*JF12*			*GY30*		
		concn (µg/L)	*RSD%*	*OAV*	*concn (µg/L)*	*RSD%*	*OAV*
ethyl hexanoate	5 (*22*)	97.60	0.68	19.5	52.17	7.79	10.4
ethyl octanoate	2 (*22*)	91.98	0.21	46.0	105.08	1.02	52.5
diethyl butanedioate	200000 (*23*)	7113.25	1.46	< 1	23715.23	3.13	< 1
fatty acids							
acetic acid	200000 (*22*)	44722.55	3.28	< 1	265223.95	6.01	1.3
2-methylpropanoic acid	200000 (*22*)	1082.06	0.75	< 1	1033.96	0.99	< 1
butanoic acid	10000 (*22*)	< q.l.	–	–	2818.68	1.86	< 1
3-methylbutanoic acid	3000 (*22*)	2546.61	0.42	< 1	5820.41	2.83	1.9
pentanoic acid	3000 (*29*)	432.07	1.09	< 1	< q.l.	–	–
hexanoic acid	3000 (*22*)	2257.73	1.01	< 1	2040.75	1.88	< 1
heptanoic acid	3000 (*29*)	< q.l.	–	–	66.22	10.26	< 1
octanoic acid	500 (*27*)	111.05	2.14	< 1	144.38	5.28	< 1
aromatic compounds							
benzaldehyde	990 (*27*)	648.15	0.47	< 1	1799.75	1.92	1.8
phenylacetaldehyde	1 (*29*)	29.01	2.45	29.0	39.17	4.56	39.2
acetophenone	65 (*27*)	59.77	0.52	< 1	274.22	2.03	4.2
ethyl benzoate	575 (*22*)	29.47	0.13	< 1	96.17	2.99	< 1

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Table 3. (Continued). Quantitative Data and OAVs of Aroma Compounds in both JF12 and GY30 (n=3)

aroma compounds	*odor threshold (µg/L)*	*JF12*			*GY30*		
		concn (µg/L)	*RSD%*	*OAV*	*concn (µg/L)*	*RSD%*	*OAV*
ethyl 2-phenylacetate	100 (*19*)	57.72	3.29	< 1	238.88	1.24	2.4
2-phenylethyl acetate	250 (*22*)	30.38	0.32	< 1	77.07	2.99	< 1
ethyl 3-phenylpropionate		< q.l.	−	−	45.69	2.65	−
2-phenylethanol	10000 (*22*)	76160.94	4.08	7.6	133472.45	21.71	13.4
phenols							
guaiacol	10 (*22*)	< q.l.	−	−	62.80	3.71	6.3
4-methylguaiacol		< q.l.	−	−	< q.l.	−	−
phenol	30 (*28*)	< q.l.	−	−	49.99	5.56	1.7
4-methylphenol	68 (*27*)	< q.l.	−	−	< q.l.	−	−
4-ethylphenol	440 (*30*)	51.68	5.31	< 1	43.51	1.47	< 1
4-vinylguaiacol	40 (*22*)	< q.l.	−	−	< q.l.	−	−
furans							
furfural	14100 (*27*)	4156.65	2.31	< 1	20532.92	1.70	1.5
2-acetylfuran		< q.l.	−		< q.l.	−	
5-methyl-2-furfural	20000 (*23*)	67.29	0.53	< 1	97.50	5.14	< 1
ethyl 2-furoate	16000 (*30*)	< q.l.	−	−	21.81	1.70	< 1

aroma compounds	*odor threshold (μg/L)*	*JF12*			*GY30*		
		concn (μg/L)	*RSD%*	*OAV*	*concn (μg/L)*	*RSD%*	*OAV*
2-furanmethanol	2000 (*31*)	< q.l.	–	–	< q.l.	–	–
lactones							
γ-nonalactone	30 (*27*)	130.36	3.76	4.4	303.64	2.95	10.1
aldehydes							
hexanal	5 (*32*)	21.16	2.38	4.2	19.14	4.43	3.8
sulfur-containing compounds							
dimethyl trisulfide	0.2 (*22*)	86.13	0.35	430.7	83.92	0.06	419.6
3-(methylthio)propanol	500 (*22*)	4233.06	2.09	8.5	31069.19	2.24	62.1
nitrogen-containing compounds							
2,5-dimethylpyrazine		< q.l.	–	–	< q.l.	–	–
2,6-dimethylpyrazine		< q.l.	–	–	< q.l.	–	–
2-acetylpyrrole	170000 (*32*)	< q.l.	–	–	48.19	12.83	< 1

[a] q.l., quantitative limit.

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Among all aroma compounds quantified in both rice wines, dimethyl trisulfide had the highest OAV, and followed by ethyl octanoate, ethyl butanoate, phenylacetaldehyde, and ethyl hexanoate with OAVs from 10 to 50. Some aroma compounds with OAVs > 10 were only detected in GY30 or JF12 rice wines, for example, 3-(methylthio)propanol (methionol), 2-phenylethanol, and γ-nonala-ctone were only detected in GY30, whereas ethyl 2-methylpropanoate only in JF12. These results were similar to the research of aroma compounds in aged sake, a kind of brewing alcoholic beverage in Japan. Isogai and co-workers (*19*) studied the changes of aroma compounds during sake aging. They showed that 3-(methylthio)propoanal (methional) and dimethyl trisulfide were present in aged sake at concentrations exceeding their odor thresholds, and the highest OAV was observed for dimethyl trisulfide. Although the formation of dimethyl trisulfide in Chinese rice wine had not been studied, it had been well-studied in beer (*20*). There were considered to be two formation pathways of dimethyl trisulfide: the reaction between methanesulfenic acid and hydrogen sulfide and the oxidation of methionol derived from the degradation of methional (*20*, *33*).

The 9 and 11 other aroma compounds in the JF12 and GY30 rice wines had OAVs from 1 to 10, respectively. These compounds mainly were short-chain alcohols, ethyl esters of fatty acids, and aromatic compounds.

In summary, GC–O is a suitable method to fast screen potent aroma compounds in Chinese rice wines. GC–O and OAVs showed that dimethyl trisulfide, phenylacetaldehyde, ethyl octanoate, and ethyl butanoate were the most important aroma compounds in Chinese rice wine. Other components, such as γ-nonalactone, 2-phenylethanol, 3-methylbutanol, and some other aromatic compounds were also determined to be powerful odorants. These odorants are associated with fruity, flora, and sweet odor descriptions, which are closed related to the aromas of Chinese rice wine.

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Chapter 17

Identification of Aroma Compounds in Chinese "Moutai" and "Langjiu" Liquors by Normal Phase Liquid Chromatography Fractionation Followed by Gas Chromatography/Olfactometry

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The aroma-active compounds in two famous Chinese soy sauce aroma type liquors, Moutai and Langjiu liquors, were investigated in this study. The aroma compounds were isolated using liquid/liquid extraction, and further fractionated into acidic, basic, and neutral fractions. The neural fraction was applied to a normal phase liquid chromatography column and further separated based on their polarity. The aroma compounds of seven fractionations were separately analyzed by GC/Olfactometry (GC/O). A total of 186 aroma-active compounds were identified by GC/O and GC/MS. Among these compounds, ethyl hexanoate, hexanoic acid, 3-methylbutanoic acid, 3-methylbutanol, 2,3,5,6-tetramethylpyrazine, ethyl 2-phenylacetate, 2-phenylethyl acetate, ethyl 3-phenylpropanoate, 4-methylguaiacol, and γ-decalactone had the highest aroma intensity. Several other basic compounds, including 2,3-dimethyl-5-ethylpyrazine, 2,3,5-trimethyl-6-ethylpyrazine, 2,3,5-trimethylpyrazine, were identified to have high aroma

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ACS Symposium Series; American Chemical Society: Washington, DC, 2012.

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indentify. In addition, vanillin, γ-heptalactone, γ-nonalactone, Z-whisky lactone, furaneol, and sotolon, were identified in the liquors. Geosmin was also detected in these two liquors.

Introduction

Chinese liquor is one of the most consumed alcoholic beverages in the world, with an annual production of more than 10 million liters in 2011 (*1*). According to aroma and flavor characteristics, Chinese liquor can be classified into 5 categories: strong aroma (Chinese named *nongxiang*), soy sauce aroma (*jiangxiang*), light aroma (*qingxiang*), sweet honey aroma (*mixiang*), and miscellaneous types. The miscellaneous type was recently further divided into 7 sub-classes, complex aroma (*jianxing*), roasted-sesame-like aroma (*zhimaxing*), herb-like aroma (*yaoxiang*), *fenxiang*, *laobaiganxiang*, *chixiang,* and *texiang* aroma types. The key important aroma of *laobaiganxing* type liquor is geosmin (*2*). The profile of aroma and flavor in *fenxiang* aroma type liquor is between strong and light aromas, and complex aroma (*jiangxiang*) between strong and soy sauce aromas. The important producing process of *chixiang* type liquor dipped pork fat into the fresh distillate, and then maturated for 20~30 days. And *texiang* type liquor has special aromas, like strong, light, and soy sauce aroma type liquor. These kinds aroma type liquor is made from special fermentation process, and not from blending.

Moutai liquor (also named Maotai liquor) is one of the most famous liquors in China, and Langjiu has a similar flavor characteristic to Moutai. Both liquors belong to soy sauce aroma type (*jiangxing*) liquor (*3–5*). They are fermented from sorghum with Daqu powder made from wheat, as a starter. The fermentation process of soy sauce aroma type liquor is different from that of strong aroma type liquor, involving 8 cycles of fermentation. In this process, sorghum is milled, mixed with water, and then cooked. The cooked grain is cooled, and mixed with appropriate Daqu powder. This mixture is stacked on the ground for 2-3 days to initate the fermentation (final temperature 40~50 °C). The grains is then moved to a special fermenter, which is a cuboid vessel made of stone, and the bottom is coated with a layer of fermentation mud made of clay, spent grain, bean cake powder, and fermentation bacteria (*Clostridium* sp.) (see Figure 1). The 1st cycle of fermentation takes 30 days. After the 1st cycle, the fermented grains is mixed with milled sorghum, and distilled with steam (see Figure 2). After distillation, the grains were cooked in the same distiller, and then repeated the 1st cycle producing process, and named 2nd cycle until fermentation finished, and distilled to gain the raw liquor. From 3rd to 8th cycle fermentation, the fermented grains each cycle, not mixed with sorghum, directly distilled by steam (see Scheme 1). These base liquors are stored in pottery jars, and aged for less than 5 years, blended,and marketed accordingly.

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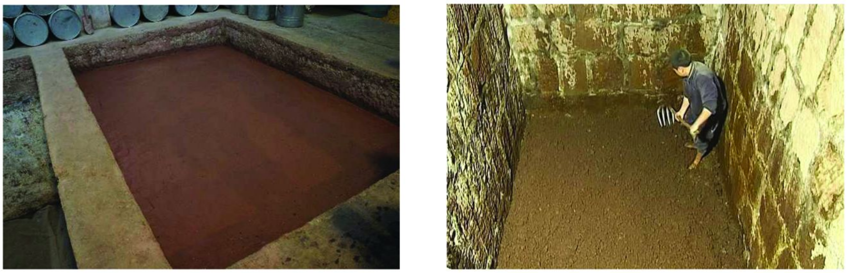


Figure 1. Fermenter for fermentation of cooked-grains.

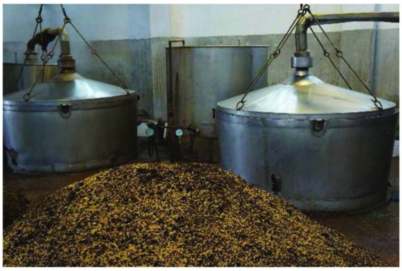
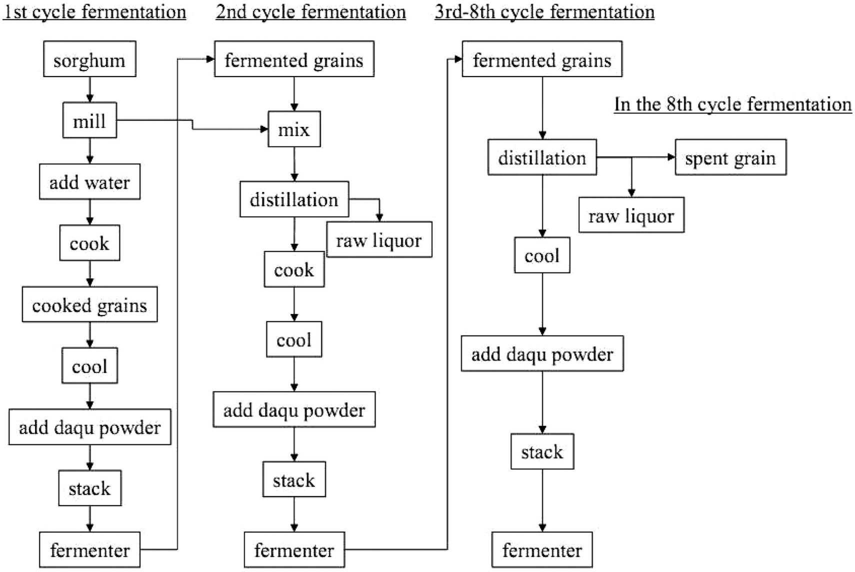


Figure 2. The distillation and fermented grains.


1st cycle fermentation
sorghum
mill
add water
cook
cooked grains
cool
add daqu powder
stack
fermenter
2nd cycle fermentation
fermented grains
mix
distillation
raw liquor
cook
cool
add daqu powder
stack
fermenter
3rd-8th cycle fermentation
fermented grains
In the 8th cycle fermentation
distillation
spent grain
raw liquor
cool
add daqu powder
stack
fermenter

Scheme 1. Producing process flowchart of soy sauce aroma type liquor

Limited literature has reported the aroma chemistry of Chinese liquor. Using GC/Olfactometry (GC/O) and GC/MS, Fan and Qian (*3*, *4*, *6*) identified the aroma-active compounds in strong aroma type Chinese liquors such as Yanghe, Wuliangye and Jiannanchun liquors, and found esters, alcohols, furans, and sulfur-containing compounds are important to strong aroma type Chinese liquor.

Very little is known about the aroma chemistry of soy sauce type Chinese liquors. Most of literatures on soy sauce aroma type liquors are focused on total acids, total esters, total fusel alcohols and other physical or chemical properties (*7*, *8*). More recently, Zhu and co-workers (*9*) characterized the volatile compounds of Moutai liquor by comprehensive two-dimensional GC-time of flight MS, and 528 components were identified. Fan and co-workers (*5*) identified 76 volatile compounds with stir bar sorptive extraction (SBSE) in 14 soy sauce type liquors. They found that the most abundant esters were ethyl 2-hydorxypropanate, ethyl acetate, ethyl hexanoate, ethyl propanoate, ethyl 2-methylpropanoate, ethyl butanoate, and ethyl pentanoate. The most abundant fusel alcohols were 3-methylbutanol and 1-hexanol. In addition, 3-methylbutanal, ethyl 2-phenylacetate, 2-phenylethanol, butanoic acid, hexanoic acid, furfural (2-furan-carboxaldehyde), were also at high concentration. However, it is still not clear which compounds contribute to the characteristic aroma of soy sauce aroma type of Chinese liquor.

The objective of this study is to identify the aroma-active compounds in soy sauce aroma type liquor, both Moutai and Langjiu liquors, through liquid/liquid extraction, followed by silica gel normal phase chromatography fractionation, and GC/O and GC/MS identification.

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Materials and Methods

Chemicals

3-Methylbutanoic acid (98%), 2-phenylethanol (99%) and 2-phenylethyl acetate (99%) were from Fluka, Inc (Shanghai, China). 2-Methylpropanoic acid (99%) was from Alfa Aesar, Inc (Beijing, China). Butanoic acid (98%), pentanoic acid (98%) and octanoic acid (98%) were from Ciyun Chemical Company (Wujiang, Jiangsu, China). Others were from Sigma–Aldrich China Co. (St. Louis, MO, U.S.A.).

Freon 11 (fluorotrichloromethane) of 99%+ purity was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI, U.S.A.). Pentane and methanol were from Mallinckrodt Baker Inc. (Phillipsburg, NJ, U.S.A.). Diethyl ether was obtained from Burdick & Jackson (Muskegon, MI, U.S.A.). Anhydrous sodium sulfate was from EMD Chemicals Inc. (Gibbstown, NJ, U.S.A.). Sodium chloride, sodium bicarbonate and sulfuric acid were obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Silica Gel 60, particle size 0.2–0.5 mm (35–70 mesh), was from EMD Chemical Inc. (Gibbstown, NJ, U.S.A.).

Sample

Moutai liquor (500 mL, 53% ethanol by volume) was bottled on June 23, 2004, at Moutai Stock Co. Ltd. in Renhuai City, Guizhou Province, China. Langjiu liquor (500 mL, 53% ethanol by volume) was bottled on April 8, 2004, at Langjiu Distillery Co. Ltd. in Luzhou City, Sichuan Province, China. Both samples were produced commercially in China and shipped to the U.S., where the samples were stored at 15 °C until analysis.

Aroma Compounds of Moutai Liquor Extraction and Fractionation

Aroma Extraction

Each liquor sample (100 mL) was diluted to 14% alcohol by volume with deionized water (cooled to 10°C) according to the procedures described previously (*10*, *11*). The diluted sample was saturate with analytical-grade sodium chloride, and then extracted three times with 100 mL aliquots of Freon 11 each in a separatory funnel. All extracts were combined and concentrated to 100 mL with a stream of nitrogen. It was labeled as 'extract 1'.

Acidic, Basic, Water-Soluble, and Neutral Fractionation

Fifty milliliters deionized water was added to the 'extract 1,' and the aqueous phase was saturated with NaCl and adjusted to pH 9 with sodium bicarbonate solution (10% w/v). The organic phase was separated in a separatory funnel and saved as 'extract 2'. The aqueous phase was adjusted to pH 1 with 2 N H_2SO_4, and extracted three times with 25 mL aliquots of freshly redistilled diethyl ether. The diethyl ether extracts were combined and dried over 5 g anhydrous sodium sulfate overnight. The extract was slowly concentrated to 2 mL in a fume hood and then to 500 μL with a stream of nitrogen. This concentrate was labeled as the 'acidic fraction.'

Fifty milliliters deionized water was added to 'extract 2' and the aqueous phase was saturated with NaCl. The pH of the aqueous phase was adjusted to 1 with 2 N H_2SO_4, and then separated in a separatory funnel and saved. The organic phase was labeled as 'extract 3.' The aqueous phase was adjusted to pH 9 with sodium bicarbonate solution (10% w/v), and then extracted three times with 20 mL aliquots of freshly distilled diethyl ether. The diethyl ether extracts were combined and dried with 5 g anhydrous sodium sulfate overnight. The extract was slowly concentrated to 2 mL and then to 500 μL with a stream of nitrogen. This concentrate was labeled as the 'basic fraction.'

'Extract 3' was washed with 20 mL deionized water one time. The organic phase was labeled as 'extract 4.' The washing was saturated with NaCl, and then extracted two times with 20 mL aliquots of freshly distilled diethyl ether. The extract was dried overnight, and slowly concentrated to 2 mL and then to 500 μL with a stream of nitrogen. This concentrate was labeled as the 'water-soluble fraction.'

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The ‘extract 4’ was dried overnight and filtered. The filtrate was slowly concentrated to 2 mL for normal-phase liquid chromatography.

Normal Phase Liquid Chromatography

A glass column (30 cm × 1 cm i.d.) packed with 15 g of silica gel was washed with 50 mL methanol, then 50 mL diethyl ether, and then conditioned with 50 mL pentane. The ‘extract 3’ (2 mL) was applied to the column. Fifty milliliters each eluting solvent, pentane (fraction I), pentane:diethyl ether(95:5, fraction II), pentane:diethyl ether (90:10, fraction III), pentane:diethyl ether (80:20, fraction IV), pentane:diethyl ether (70:30, fraction V), and diethyl ether (fraction VI), were sequentially applied to elute the compounds from the column at a flow rate of 0.9 mL/min. All elutes were slowly concentrated to 10 mL and then to 500 μL with a stream of nitrogen for GC/O and GC/MS analysis.

GC/O Analysis

GC/O analysis was performed on a Hewlett-Packard 5890 gas chromatography (Agilent Technology, Santa Clara, CA, U.S.A.) equipped with a flame ionization detector (FID) and an olfactometer. The column carrier gas was nitrogen at constant pressure (15 p.s.i., 2 mL/min column flow measured at 25 °C). Half of the column flow was directed to the FID, while the other half was directed to the olfactometer. Samples were analyzed on a DB-Wax column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA, U.S.A.) and a HP-5 column (30 m × 0.32 mm i.d., 0.25 μm film thickness; Agilent Technology, Santa Clara, CA, U.S.A.). A 0.5 μL sample was injected into GC with splitless mode. The oven temperature was held at 40 °C for 2 min, then raised to 230 °C at a rate of 6 °C/min, and held at 230 °C for 15 min on a DB-Wax column, while the final temperature was 250 °C for 5 min on a HP-5 column. The injector and detector temperatures were 250 °C.

Two panelists (one female and one male) were selected for the GC/O study. One panelist had more than 5 years of sensory analysis experience in Chinese liquor. Both panelists were familiar with GC/O technique and had more than 100 h of training. Panelists responded to the aroma intensity of the stimulus by using a 16-point scale ranging from 0 to 15. ‘0’ was none, ‘7’ was moderate, while ‘15’ was extreme. The retention time, intensity value and aroma descriptor were recorded. Each fraction was replicated three times by each panelist. The intensity values for aroma were averaged for the three analyses. When a panelist could not detect a aroma compound, the intensity was considered as zero in the averaging process (*12*, *13*).

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Retention Indices (RI)

RIs were calculated in accordance with a modified Kovats method (*14*). A standard mixture of paraffin homologues C5–C25 was prepared. The sample and the hydrocarbon standard mixture were co-injected into the GC, and the retention times were used to calculate retention indices.

GC/MS Analysis

Capillary GC/MS was carried out using an Agilent GC 6890-5973MSD. The sample was analyzed on a DB-Wax column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and a HP-5 column (30 m × 0.32 mm i.d., 0.25 μm film thickness). The oven and injector temperatures were identical to those of GC/O analysis, described above. The column carrier gas was helium at a constant flow rate of 2 mL/min. An Agilent 5973 Mass Selective Detector (MSD) was used for identification. The electron impact (EI) energy was 70 eV, and the ion source temperature was set at 230°C. Mass spectra of unknown compounds were compared with those in the Wiley 275 Database (Agilent Technologies Inc.). Positive identification was achieved by comparing mass spectrum, aroma and retention index with those of the standard. Tentative identification was achieved by comparing aroma or mass spectrum only.

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Results and Discussion

One hundred and eighty-six aroma compounds were detected by GC/O and GC/MS in Moutai and Langjiu liquors. Of which, 184 aromas were identified or tentatively identified by GC/MS, including 18 alcohols, 1 aldehydes, 4 acetals, 13 ketones, 14 acids, 37 esters, 32 aromatic compounds, 11 phenols, 11 furanic compounds, 5 sulfur-containing compounds, 20 pyrazines, 12 lactones, and 6 miscellaneous compounds; and 2 compounds were unknown.

Fatty Acids

A total of 14 fatty acids was detected by GC/O in the acidic fraction of Moutai and Langjiu, including C2-C10 straight-chain fatty acids, and branched-chain fatty acids such as 2-methylpropanoic acid, 3-methylbutanoic acid, 4-methyl-pentanoic acid, and 5-methylhexanoic acid (tentatively identified) (Table I).

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Fatty acids gave acidic, vinegar, sweat, and rancid aromas. Among these, the fatty acids with the highest aroma intensity were hexanoic acid (intensity 10~15), 3-methylbutanoic acid (intensity 10~15), butanoic acid (intensity 14), and acetic acid (intensity 10~12). The intensities of other acids were less than 10. Both hexanoic and butanoic could be very important to the aroma of Moutai and Langjiu because the concentration of hexanoic and butanoic acids in Langjiu liquor has been reported to be in the range of 28~218 mg/L and 40~140 mg/L, respectively (*5*), and their odor thresholds were 2.5 mg/L for hexanoic acid and 1 mg/L for butanoic acid in 46% hydroalcoholic solution (*15*). Hexanoic and butanoic acids were produced by fermentation bacteria (*Clostridium* sp.) in fermentation mud (*3*, *4*, *16*).

Higher Alcohols

Eighteen alcohols were detected by GC/O in both liquors, including 1-propanol, 2-methylpropanol, 1-butanal, 2-butanol, 3-methylbutanol, 2-methyl-butanol, 1-pentanol, 2-pentanol, 1-hexanol, 1-heptanol, 2-heptanol, 3-octanol, and 1-octen-3-ol. Most of alcohols distributed in acidic, basic, water-soluble, and fraction IV fractions (Table I, III, and VI).

The most important alcohol was 3-methylbutanol, and it had an aroma intensity of 13 in fraction IV, 8~10 in fraction V+VI, 2~6 in acidic fraction, and 3 in water-soluble fraction in both liquors. 3-Methylbutanol gave nail polish aromas. The level of this alcohol was 58~628 mg/L in Langjiu liquor (*5*), and its threshold was 179.mg/L in 46% hydroalcoholic solution (*15*).

Other alcohols identified were 2-butanol, 2-methylpropanol, 2-pentanol, 1-pentanol, 1-hexanol, 3-octanol, 1-nonanol, and 1-octen-3-ol, and the intensities of these alcohols were all less than 10 in different fractions. Of these, the concentrations of 1-propanol, 1-hexanol, and 1-heptanol were more than 1 mg/L, while the concentrations of other alcohols were less than 1 mg/L (*5*, *8*). It has been reported that the concentration of 1-octen-3-ol ranged from 107 to 362 μg/L in Langjiu liquor (*5*), and its odor threshold was 6 μg/L in 46% ethanol-water solution (*15*). Based on its concentration and low odor threshold, 1-octen-3-ol would contribute to the overall aroma of soy sauce aroma type liquors.

Ketones

Thirteen ketones were identified by GC/O in both liquors, mainly detected in fraction II (Table IV). Some ketones were existed in both basic and water-soluble fractions (Table II). The intensities of most ketones were less than 10. Ketones gave fruity, berry, buttery, and cream aromas. 2-Hydroxy-3-hexanone was tentatively identified by MS in this study.

Pyrazines

In this study, 20 pyrazines were identified by GC/O in the basic fraction, and fraction V+VI, and IV (Table III, VI and VII, Figure 3). Of which, 2-methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine,

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2,3-dimethyl-pyrazine, 2-ethylpyrazine, 2-ethyl-6-methylpyrazine, 2-ethyl-5-methylpyrazine, 2,3,5-trimethylpyrazine, 2,6-diethylpyrazine, 2,5-dimethyl-3-ethylpyrazine, 2,3-dimethyl-5-ethylpyrazine, 2,3,5,6-tetramethylpyrazine, 2,3,5-trimethyl-6-ethylpyrazine, 2-vinyl-6-methylpyrazine, and 2,3-dimethyl-*Z*-5-propenylpyrazine have been detected in Chinese liquors previously (*3*, *17*). 2,5-Dimethyl-3-butylpyrazine, 3,5-dimethyl-2-pentylpyrazine, 2-(3-methylbutyl)-6-methylpyrazine (tentatively identified), 2-methoxy-3-butyl-pyrazine (tentatively identified), and 3-(1-methylethyl)-2-methoxypyrazine (tentatively identified) were first detected in Chinese liquor. A sample charomatogram of the basic fraction is illustrated in Figure 3.

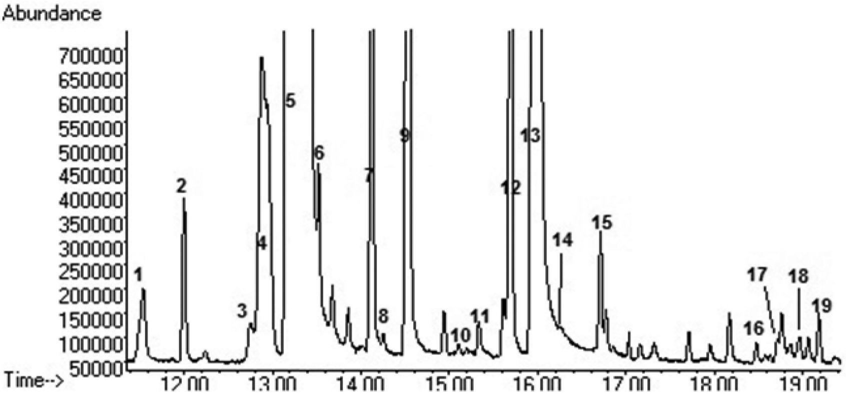

Figure 3. Pyrazines in basic fraction of Moutai liquor detected by GC/MS on polar column. Key: 1) 2-methylpyrazine; 2) 3-hydroxy-2-butanone; 3) 2,5-dimethylpyrazine; 4) 2,6-dimethylpyrazine; 5) ethyl 2-hydroxypropanoate; 6) 2,3-dimethylpyrazine; 7) 2-ethyl-6-methylpyrazine; 8) 2-ethyl-5-methylpyrazine; 9) 2,4,5-trimethylpyrazine; 10) 2,6-diethylpyrazine; 11) 2,5-dimethyl-3-ethylpyrazine; 12) 2,3-dimethyl-5-ethylpyrazine; 13) furfural; 14) 2,3,5,6-tetramethylpyrazine; 15) 2,3,5-trimethyl-6-ethylpyrazine; 16) 2-butyl-3,5-methylpyrazine; 17) 2-acetylpyridine; 18) 2-acetyl-5-methylfuran; and 19) 2-acetyl-6-methylpyridine.

2,3,5,6-Tetramethylpyrazine had overall the highest aroma intensity (intensities 4~10 in basic fraction, 12 in fraction IV, and 4~7 in fraction V+VI), followed by 2,3-dimethyl-5-ethylpyrazine, 2,3,5-trimethyl-6-ethylpyrazine, 2,3,5-trimethylpyrazine, 2-methoxy-3-butylpyrazine (tentatively identified), 3-(1-methylethyl)-2-methoxypyrazine (tentatively identified), 2-(3-methylbutyl)-6-methylpyrazine (tentatively identified), and 2,3-dimethyl-Z-5-propenylpyrazine (tentatively identified) (intensity $\geq$ 10). Pyrazines contributed to roasted and baked aromas, and methoxypyrazines gave vegetable-like aroma. The number of pyrazines and their concentrations in soy sauce aroma liquors were much higher than strong aroma, light aroma, and other aroma type Chinese liquors (*17*).

Pyrazines had a wide range of odor threshold. 2-methylpyrazine has a high odor threshold of 122 mg/L, 2,3-dimethylpyrazine 11 mg/L, 2,5-dimethylpyrazine 3 mg/L, 2,6-dimethylpyrazine 0.79 mg/L, 2-ethylpyrazine 22 mg/L, 2,3,5-trimethyl-pyrazine 0.73 mg/L, and 2,3,5,6-tetramethylpyrazine 80 mg/L in 46% hydroalcoholic solution (*15*).

Pyrazines can be produced by the Maillard reaction between saccharide and amino residues (*18*). They can also be generated from metabolic activities of microorganisms (*19*, *20*). It has been reported that 2,3,5,6-tetramethylpyrazine can be formed from 3-hydroxy-2-butanone in the presence of ammonium phosphate by *Bacillus subtilis* in Chinese liquor fermentation process (*21–23*).

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Esters

Ester was one of the most important classes of aroma compounds in Chinese liquors including soy sauce aroma type liquor. Thirty-seven esters were identified by GC/O and GC/MS in fraction II (Table IV), mainly ethyl and acetate esters.

Ethyl hexanoate had an aroma intensity of 13~15 in fraction II. Ethyl hexanoate is the key aroma compound of strong aroma type liquor (*3*, *4*, *6*). The concentration of ethyl hexanoate in Langjiu liquor was reported in the range of 246~1293 mg/L (*5*), and its threshold was 55 μg/L in 46% hydroalcoholic solution (*15*).

Other esters had high aroma intensity (intensity ≥ 10) were ethyl butanoate, ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, ethyl pentanoate, ethyl 2-methylpentanoate, and ethyl 2-hydroxyhexanoate (detected in fraction IV). These esters had been detected in Chinese liquors (*3*, *4*, *6*). Esters are the major fermentation products from fungi and yeast (*24*).

Aldehydes and Acetals

Only 1 aldehydes, 3-methylbutanal, was detected by GC/O in both liquors (Table IV). It was green and malt aromas, and its intensity was 4~5. In previous research, the concentration of 3-methylbutanal was 38~426 mg/L in Langjiu liquor (*5*), and its threshold was 16 μg/L in 46% hydroalcoholic solution (*15*). It should be an important aroma contributor based on the odor activity value (OAV, ratio of concentration to odor threshold value).

Four acetals were found in fraction II of soy sauce aroma type liquors, including 1,1-diethoxyethane, 1,1-diethoxy-2-methylpropane, 1,1-diethoxy-3-methylbutane, and 1-ethoxy-1-propoxyethane (tentatively identified) (Table IV). Acetals gave fruity aroma, and had low aroma intensity (≤ 10) except for 1,1-ethoxyethane (4~11 in fraction II). Acetals were from the reaction of aldehydes with alcohols. During the long aging process, aldehydes will condense with alcohols to form more acetals.

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Table I. Aroma compounds in the acidic fraction detected by GC/MS and GC/O on DB-Wax column

odor No.	*RI*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
					MT	*NJ*
12	1020	2-butanol	fruity	MS, aroma, RI	ND	2
14	1035	1-propanol	ripe, fruity	MS, aroma, RI	ND	3
19	1087	2-methylpropanol	wine, solvent	MS, aroma, RI	ND	6
20	1114	2-pentanol	fruity	MS, aroma, RI	1	ND
28	1201	3-methylbutanol	rancid, nail polish	MS, aroma, RI	2	6
29	1220	2-methylbutanol	rancid, nail polish	MS, aroma, RI	ND	4
31	1268	1-pentanol	fruity, balsamic	MS, aroma, RI	ND	1
43	1334	ethyl 2-hydroxypropanoate	fruity	MS, aroma, RI	ND	2
60	1424	acetic acid	acidic, vinegar	MS, aroma, RI	10	12
68	1456	2-furancarboxaldehyde	sweet, almond	MS, aroma, RI	ND	1
75	1525	propanoic acid	vinegar	MS, aroma, RI	6	5
80	1555	2-methylpropanoic acid	acid, rancid	MS, aroma, RI	8	5
91	1602	butanoic acid	rancid, cheesy	MS, aroma, RI	14	14
100	1655	3-methylbutanoic acid	rancid, acidic	MS, aroma, RI	15	10
104	1695	dihydro-2(3H)-furanone	coconut	MS, aroma, RI	8	4
109	1727	pentanoic acid	sweaty, rancid	MS, aroma, RI	8	4

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Table I. (Continued). Aroma compounds in the acidic fraction detected by GC/MS and GC/O on DB-Wax column

odor No.	*RI*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
					MT	*NJ*
116	1794	4-methylpentanoic acid	rancid, sweaty	MS, aroma, RIL	4	ND
121	1846	hexanoic acid	sweaty, cheesy	MS, aroma, RI	15	10
125	1871	5-methylhexanoic acid*	cheesy, sweaty	MS, aroma	4	ND
127	1906	2-phenylethanol	rosy, honey	MS, aroma, RI	7	5
132	1955	heptanoic acid	rancid, unpleasant	MS, aroma, RI	5	7
139	2018	dihydro-5-pentyl-2(3H)-furanone	sweet, cocoa	MS, aroma, RI	10	ND
143	2060	octanoic acid	sweat, cheesy	MS, aroma, RI	6	4
148	2168	nonanoic acid	unpleasant, fatty	MS, aroma, RI	5	6
151	2204	3-hydroxy-4,5-dimethyl-2(5H)-furanone	sweet, floral, spicy	MS, aroma, RI	10	10
154	2282	decanoic acid	fatty, unpleasant	MS, aroma, RI	ND	7
155	2308	2,6-dimethoxyphenol	smoky	MS, aroma, RI	4	5
158	2449	benzoic acid	floral, fruity	MS, aroma, RI	6	5
160	2555	2-phenylacetic acid	fruity, rosy	MS, aroma, RI	3	6
161	2559	4-hydroxy-3-methoxybenzaldehyde	sweet, floral, vanillin	MS, aroma, RI	12	14

odor No.	RI	aroma compounds	descriptor	basic of identification[a]	intensity	
					MT	NJ
162	2619	phenylpropanoic acid*	fruity, floral	MS, aroma	3	5
163	2748	phenylbutanoic acid*	floral, fruity	MS, aroma	4	ND

[a] MS: Compounds were identified by MS spectra. Aroma: Compounds were identified by the aroma descriptors. RI: Compounds were identified by comparison to pure standard. RIL: Compounds were identified by comparison with retention index from the literatures. * tentatively identified on DB-Wax column. ND: not detected by GC/O.

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Table II. Aroma compounds in the water-soluble fraction detected by GC/MS and GC/O on DB-Wax column

odor No.	*RI*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
					MT	*NJ*
1	892	ethyl acetate	pineapple	MS, aroma, RI	4	3
2	905	1,1-diethoxyethane	fruity	MS, aroma, RI	7	5
9	972	2-pentanone	fruity	MS, aroma, RI	5	6
12	1020	2-butanol	fruity	MS, aroma, RI	3	4
14	1035	1-propanol	ripe, fruity	MS, aroma, RI	ND	2
19	1087	2-methylpropanol	wine, solvent	MS, aroma, RI	ND	1
20	1114	2-pentanol	fruity	MS, aroma, RI	3	4
22	1137	1-butanol	fruity, alcoholic	MS, aroma, RI	2	ND
28	1201	3-methylbutanol	rancid, nail polish	MS, aroma, RI	3	3
31	1268	1-pentanol	fruity, balsamic	MS, aroma, RI	6	ND
35	1293	3-hydroxy-2-butanone	cream, buttery	MS, aroma, RI	4	2
43	1334	ethyl 2-hydroxypropanoate	fruity	MS, aroma, RI	4	2
68	1456	2-furancarboxaldehyde	sweet, almond	MS, aroma, RI	6	ND
72	1501	benzaldehyde	fruity, berry	MS, aroma, RI	5	2
76	1527	ethyl 2-hydroxyhexanoate	floral, jasmine	MS, aroma, RIL	4	5
83	1572	5-methyl-2-furfural	green, roasted	MS, aroma, RI	4	ND
99	1647	2-furanmethanol	baked	MS, aroma, RI	4	4

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odor No.	*RI*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
					MT	*NJ*
127	1906	2-phenylethanol	rosy, honey	MS, aroma, RI	ND	2
151	2204	3-hydroxy-4,5-dimethyl-2(5H)-furanone	sweet, floral, spicy	MS, aroma, RI	2	6

[a] MS: Compounds were identified by MS spectra. Aroma: Compounds were identified by the aroma descriptors. RI: Compounds were identified by comparison to pure standard. RIL: Compounds were identified by comparison with retention index from the literatures. ND: not detected by GC/O.

Furanic Compounds

Eleven furans were found in fraction III and IV of both Chinese liquors, for example, 2-acetylfuran, 2-acetyl-5-methylfuran, 2-furancarboxaldehyde (furfural), 5-methylfurfural, 2-furanmethanol, 2-furaldehyde diethyl acetal, 2-furfuryl ethyl ether, ethyl 2-furoate, 1-(2-furanyl)-1-propanone (tentatively identified), 1-(2-furanyl)-1-butanone (tentatively identified), and 1-(5-methyl-2-furanyl)-1-propanone (tentatively identified) (Table V **and** VI).

One of the important furanic compounds could be furfural, its intensities were 5~10 in fraction III, 0~8 in fraction V+VI, 0~6 in water-soluble fraction, 0~1 in acidic fraction, and 0~2 in basic fraction. It had a sweet and almond aroma. Furfural had an odor threshold was 44 mg/L in 46% hydroalcoholic solution (*15*). Furfural could be generated from hydrolysis of pentosan at high temperature and low pH (about 3.4) conditions during the fermentation and distillation stages (*25*), its concentration in liquor increases with distillation time (*26*). The concentration of furfural in Chinese liquors is regulated to be less than 0.4 g/L by the Chinese Liquor Industry (*7*), but usually controlled in 4~484 μg/L at present (*5*).

Several other furanic compounds were identified. 2-Acetyl-5-methylfuran also had high odor intensities (0~10 in fraction IV and 0~4 in fraction III). Its concentration was in the range of 239~1142 μg/L in soy sauce aroma type liquor (*5*), and has an odor threshold of 40 mg/L in 46% hydroalcoholic solution (*15*). 2-Furfuryl ethyl ether was first detected by GC/O in Chinese liquor, and it gave fruity aroma, and had low aroma intensity. 2-Furfuryl ethyl ether is fromed from ethanol and furfuryl alcohol, and is an important aroma contributor in age beer (*27*). Furanmethanol was also detected by GC/O in almost all fractions of both Chinese liquors, but its odor intensity was less than 10.

Benzoic Compounds

Thirty-two benzoic compounds were detected and identified by GC/O and GC/MS in both liquors, including acids, alcohols, aldehydes, ketones, and esters. Most of these existed in fraction II (Table IV), and gave rose, floral, fruity, honey, and sweet aromas.

Among these compounds, ethyl 2-phenylacetate, 1,2-dimethoxy-3-methylbenzene, 2-phenylethyl acetate, and ethyl 3-phenylpropanoate had high aroma intensities. Of these, 1,2-dimethoxy-3-methylbenzene was first detected in Chinese liquor. The concentrations of ethyl 2-phenylacetate, 2-phenylethyl acetate, and ethyl 3-phenylpropanote were 5~46 mg/L, 37~158 μg/L, and 408~1269 μg/L, respectively, in Langjiu liquors. The odor thresholds of these three compounds were 406, 908, and 125 μg/L in 46% ethanol-water solution, respectively (*15*).

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2-Phenylethyl butanoate and 2-phenylethanol also had high aroma intensity (>10). 2-Phenylethanol was detected in all Chinese liquor (*8*). In soy sauce aroma type liquor, its concentration was 7~14 mg/L, and its odor threshold was 29 mg/L in 46% hydroalcoholic solution (*15*).

Other benzoic compounds identified included 2-phenylethyl butanoate, 1,2-dimethoxybenzene, 1-phenyl-1-ethanol, phenylpropanol, 3-phenyl-2-propenal, 4-(4-methoxyphenyl)-2-butanone (bramble ketone), and 2-aminoacetophenone. 2-Aminoacetophenone was first detected in Chinese liquor. It gives shoe insole and mothball off-flavor in wine (*28*, *29*).

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Volatile Phenols

Most of phenolic compounds were detected in fractions III, IV, and V+VI of two soy sauce aroma type liquors.

4-Methyl-2-methoxyphenol (4-methylguaiacol) could be an important aroma compound in both liquors, its intensities were 0~15 in fraction IV and 7~9 in fraction III, and odor threshold was 314 μg/L in 46% ethanol-water solution (*15*).

Other aroma-active compounds (intensity $\geq$ 10) were 4-methylphenol, 4-ethylphenol, and 4-hydroxy-3-methoxybenzaldehyde (vanillin). 4-Methylphenol has phenol and animal aromas, and 4-ethylphenol gave smoky aroma. Their concentrations were 160~3002 μg/L and 86~130 μg/L in Langjiu liquors (*5*), and threshold 160 μg/L and 618 μg/L in 46% hydroalcoholic solution, respectively (*15*). 4-Methylphenol and 4-ethylphenol can cause off-flavor of wine at high concentration, especially 4-ethylphenol (*29*). Vanillin was detected by GC/O in Chinese liquor, and it gave sweet, floral, and vanillin aromas. Its odor threshold was 438 μg/L in 46% ethanol-water solution (*15*). Volatile phenols could be from the metabolism of ferulic acid and *p*-cumaric acid by yeasts during the fermentation (*30*).

Sulfur-Containing compounds

In this study, 5 sulfur-containing compounds were detected by GC/O, including dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide, benzothiazole, and methyl thio furoate (tentatively identified).

Benzothiazole has a smoky and rubber-like aroma, and other sulfur-containing compounds gave sulfur, onion, cabbage, and rotten cabbage aromas. These compounds had been detected in Chinese liquor except for methyl thio furoate which was tentatively identified (*3*, *4*). In 46% ethanol-water solution, the odor threshold of dimethyl disulfide and dimethyl trisulfide were 9 and 0.36 μg/L, respectively (*15*). Sulfur-containing compounds could come from the degradation of sulfur-containing amino acids during fermentation (*31*).

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Table III. Aroma compounds in the basic fraction detected by GC/MS and GC/O on DB-Wax and HP-5 columns

odor No.	*RI_{Wax}*	*$RI_{HP\text{-}5}$*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
11	989	556	2,3-butanedione	buttery, cream	MS, aroma, RI	4	4
14	1035	530	1-propanol	ripe, fruity	MS, aroma, RI	3	ND
27	1193	861	2,4,5-trimethyloxazole	green, baked, musty	MS, aroma, RI	4	5
32	1276	801	2-methylpyrazine	green, hazelnut	MS, aroma, RI	3	ND
35	1293	717	3-hydroxy-2-butanone	cream, buttery	MS, aroma, RI	ND	2
38	1315	915	2,5-dimethylpyrazine	baked, nut	MS, aroma, RI	1	1
42	1330	910	2,6-dimethylpyrazine	baked, nut	MS, aroma, RI	4	4
47	1350	923	2,3-dimethylpyrazine	baked, nut	MS, aroma, RI	4	ND
49	1375	981	2-ethyl-6-methylpyrazine	nut, roasted	MS, aroma, RI	4	ND
53	1385		2-ethyl-5-methylpyrazine	baked, roasted	MS, aroma, RI	1	ND
55	1400	1000	2,3,5-trimethylpyrazine	roasted, nut	MS, aroma, RI	8	6
59	1415	1030	2,6-diethylpyrazine	nut, baked	MS, aroma, RI	6	ND
61	1425	973	ethyl 2-hydroxy-3-methylbutanoate	cut grass, fruity	MS, aroma, RIL	6	ND
64	1430	1080	2,5-dimethyl-3-ethylpyrazine	roasted, baked	MS, aroma, RI	3	ND
66	1445	1088	2,3-dimethyl-5-ethylpyrazine	baked	MS, aroma, RI	9	10
68	1456	831	2-furancarboxaldehyde	sweet, almond	MS, aroma, RI	2	ND
69	1460	1093	2,3,5,6-tetramethylpyrazine	roasted, baked	MS, aroma, RI	10	4

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odor No.	*RI_{Wax}*	*RI_{HP-5}*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
73	1509	1163	2,3,5-trimethyl-6-ethylpyrazine	baked	MS, aroma, RI	4	10
82	1562	1208	2-methoxy-3-butylpyrazine***	vegetable-like	aroma, RIL	4	10
83	1572	967	5-methyl-2-furfural	green, roasted	MS, aroma, RI	4	10
85	1583	1263	2,5-dimethyl-3-butylpyrazine	baked	MS, aroma, RIL	ND	4
90	1598	1026	2-acetylpyridine	popcorn, cooked rice	MS, aroma, RI	ND	5
99	1647	854	2-furanmethanol	baked	MS, aroma, RI	4	4
127	1906	1116	2-phenylethanol	rosy, honey	MS, aroma, RI	6	4
151	2204	1120	3-hydroxy-4,5-dimethyl-2(5H)-furanone	sweet, floral, spicy	MS, aroma, RI	ND	4
165		878	4-ethoxy-2-butanone**	fruity	MS, aroma	4	ND
166		903	4-ethoxy-2-pentanone**	fruity, green	MS, aroma	2	3

[a] MS: Compounds were identified by MS spectra. Aroma: Compounds were identified by the aroma descriptors. RI: Compounds were identified by comparison to pure standard. RIL: Compounds were identified by comparison with retention index from the literatures. *: tentatively identified on DB-Wax column. ** tentatively identified on HP-5 column. *** tentatively identified on DB-Wax and HP-5 columns. ND: not detected by GC/O.

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Table IV. Aroma compounds in the neutral fraction II detected by GC/MS and GC/O on DB-Wax and HP-5 columns

odor No.	*RI_{Wax}*	*RI_{HP-5}*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
1	892	581	ethyl acetate	pineapple	MS, aroma, RI	2	ND
2	905	739	1,1-diethoxyethane	fruity	MS, aroma, RI	4	11
3	915	628	3-methylbutanal	green, malt	MS, aroma, RI	4	5
5	953	714	ethyl propanoate	banana, fruity	MS, aroma, RI	ND	5
6	961	761	ethyl 2-methylpropanoate	fruity, sweet	MS, aroma, RI	10	8
7	965	812	1-ethoxy-1-propoxyethane*	fruity	MS, aroma	ND	5
8	969	859	1,1-diethoxy-2-methylpropane	fruity	MS, aroma, RI	8	6
10	988		2-methylpropyl acetate	floral, fruity	MS, aroma, RI	3	ND
13	1031	800	ethyl butanoate	pineapple, fruity	MS, aroma, RI	12	13
15	1045	849	ethyl 2-methylbutanoate	berry, fruity	MS, aroma, RI	10	12
16	1060	852	ethyl 3-methylbutanoate	apple	MS, aroma, RI	12	12
18	1068	955	1,1-diethoxy-3-methylbutane	fruity	MS, aroma, RI	8	4
21	1145	900	ethyl pentanoate	apple	MS, aroma, RI	12	14
25	1178	928	methyl hexanoate	floral, fruity	MS, aroma, RI	ND	5
26	1182	969	ethyl 4-methylpentanoate	fruity, floral	MS, aroma, RI	8	6
30	1235	1008	ethyl hexanoate	fruity, floral, sweet	MS, aroma, RI	13	15
33	1275		hexyl acetate	fruity, floral	MS, aroma, RI	1	ND

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odor No.	RI_{Wax}	RI_{HP-5}	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
34	1284		2-furfuryl ethyl ether	fruity	MS, aroma, RIL	6	ND
41	1319	1094	propyl hexanoate	fruity	MS, aroma, RI	4	1
44	1336	1097	ethyl heptanoate	fruity	MS, aroma, RI	5	7
46	1346	1152	3-methylbutyl pentanoate	fruity, floral	MS, aroma, RI	4	ND
48	1360	976	dimethyl trisulfide	sulfur, rotten cabbage	MS, aroma, RI	ND	5
50	1380		2-nonanone	sweet, berry, fruity	MS, aroma, RI	3	ND
52	1384	1189	butyl hexanoate	pineapple, fruity	MS, aroma, RI	5	6
56	1404	1135	ethyl cyclohexanecarboxylate	floral, fruity	MS, aroma, RIL	5	ND
57	1409	1196	ethyl octanoate	fruity	MS, aroma, RI	3	5
62	1429	1250	3-methylbuty hexanoate	fruity, apple, green	MS, aroma, RI	6	3
72	1501	963	benzaldehyde	fruity, berry	MS, aroma, RI	ND	7
74	1509	1298	ethyl nonanoate	floral, fruity	MS, aroma, RI	ND	5
79	1554	1349	3-methylbutyl heptanoate	fruity	MS, aroma, RI	6	ND
84	1583	1386	hexyl hexanoate	apple, peach	MS, aroma, RI	4	ND
93	1610	1394	ethyl decanoate	fruity, grape	MS, aroma, RI	4	3
94	1620	1046	phenyl acetaldehyde	fruity	MS, aroma, RI	ND	2
97	1640	1175	ethyl benzoate	herb, fruity	MS, aroma, RI	3	8

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Table IV. (Continued). Aroma compounds in the neutral fraction II detected by GC/MS and GC/O on DB-Wax and HP-5 columns

odor No.	*RI_{Wax}*	*RI_{HP-5}*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
103	1690	1328	1,1-diethoxy-2-phenylethane	floral, fruity	MS, aroma, RI	4	ND
108	1725		benzyl acetate	sweet candy, fruity	MS, aroma, RI	2	2
113	1757		methyl 2-phenylacetate	fruity	MS, aroma, RI	ND	2
114	1768	1247	ethyl 2-phenylacetate	rosy, honey	MS, aroma, RI	14	11
117	1801	1261	2-phenylethyl acetate	rosy, floral	MS, aroma, RI	3	2
119	1828	1563	ethyl dodecanoate	sweet, fruity	MS, aroma, RI	4	ND
122	1853		propyl 2-phenylacetate*	rosy, honey	MS, aroma	6	ND
126	1872	1354	ethyl 3-phenylpropanoate	fruity, floral, rosy	MS, aroma, RI	12	12
130	1950		unknown	roasted		ND	8
133	1958	1445	2-phenylethyl butanoate	floral, rosy	MS, aroma, RI	4	10
137	2005		ethyl phenylbutanoate*	floral, fruity	MS, aroma	5	ND
138	2009		2-pentadecanone	fruity	MS, aroma, RI	5	ND
141	2027		4-hydroxy-2,5-dimethyl-3(2H)-furanone	strawberry	MS, aroma, RI	ND	2
144	2066		5-methyl-2-phenyl-2-hexenal*	fruity, sweet	MS, aroma	4	4
164		875	3-methylbutyl acetate	sweet, fruity, apple	MS, aroma, RI	5	ND
168		941	2-methylpropyl butanoate	fruity, sweet	MS, aroma, RI	2	1

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odor No.	*RI_{Wax}*	*$RI_{HP\text{-}5}$*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
169		943	ethyl 2-methylpentanoate	apple, fruity	MS, aroma, RI	10	10
172		991	2-octanone	blue cheese	MS, aroma, RI	4	4
174		1040	2-methylpropyl 3-methylbutanoate**	sweet, fruity	MS, aroma	5	ND
175		1055	3-methylbutyl butanoate	floral, fruity	MS, aroma, RI	2	ND
177		1150	methyl thio furoate**	sulfide	MS, aroma	4	5
180		1290	pentyl hexanoate	sweet, fruity	MS, aroma, RI	ND	4
181		1424	1-phenyl-1-hexanone**	rosy	MS, aroma	8	5
182		1454	geranyl acetone	rosy, fruity	MS, aroma, RI	ND	6
183		1509	ethyl E,Z-2,4-decadienoate	sweet, fruity	MS, aroma, RI	1	6
184		1545	2-phenylethyl 3-methylbutanoate**	fruity, woody	MS, aroma	ND	5

[a] MS: Compounds were identified by MS spectra. Aroma: Compounds were identified by the aroma descriptors. RI: Compounds were identified by comparison to pure standard. RIL: Compounds were identified by comparison with retention index from the literatures. * tentatively identified on DB-Wax column. ** tentatively identified on HP-5 column. ND: not detected by GC/O.

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Table V. Aroma compounds in the neutral fraction III detected by GC/MS and GC/O on DB-Wax and HP-5 columns

odor No.	*RI_{Wax}*	*RI_{HP-5}*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
1	892	581	ethyl acetate	pineapple	MS, aroma, RI	3	8
23	1170	896	2-heptanone	green, berry, fruity	MS, aroma, RI	4	2
24	1175		cyclopentanone*	fruity	MS, aroma	2	ND
36	1300	980	1-octen-3-one	mushroom	MS, aroma, RI	4	ND
43	1334	815	ethyl 2-hydroxypropanoate	fruity	MS, aroma, RI	ND	5
51	1382		3-octanol	green, fruity	MS, aroma, RI	2	5
54	1387		unknown	fruity		ND	7
55	1400	1000	2,3,5-trimethylpyrazine	roasted, nut	MS, aroma, RI	ND	10
61	1425	973	ethyl 2-hydroxy-3-methylbutanoate	cut grass, fruity	MS, aroma, RI	6	4
67	1448	986	1-octen-3-ol	mushroom	MS, aroma, RI	3	2
68	1456	831	2-furancarboxaldehyde	sweet, almond	MS, aroma, RI	5	10
70	1489	917	2-acetylfuran	sweet, caramel	MS, aroma, RI	3	2
71	1495		2-decanone	berry, fruity	MS, aroma, RI	4	2
72	1501	967	benzaldehyde	fruity, berry	MS, aroma, RI	3	4
76	1527	1068	ethyl 2-hydroxyhexanoate	floral, jasmine	MS, aroma, RIL	6	6
81	1557	1011	1-(2-furanyl)-1-propanone*	fruity, sweet	MS, aroma	5	ND
83	1572	967	5-methyl-2-furfural	green, roasted	MS, aroma, RI	3	ND

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odor No.	*RI$_{Wax}$*	*RI$_{HP-5}$*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
87	1589		1-ethyl-2-formylpyrrole*	baked	MS, aroma	4	2
89	1593	1041	2-acetyl-5-methylfuran	roasted	MS, aroma, RI	4	ND
92	1603	1058	ethyl 2-furoate	balsamic	MS, aroma, RI	4	ND
94	1620	1046	phenylacetaldehyde	floral, rosy	MS, aroma, RI	4	5
95	1625	1035	acetophenone	sweet, fruity, floral	MS, aroma, RI	4	4
96	1632	1102	1-(2-furanyl)-1-butanone*	fruity	MS, aroma	ND	4
99	1647	854	2-furanmethanol	baked	MS, aroma, RI	5	3
101	1655	1186	diethyl butanedioate	fruity, sweet	MS, aroma, RI	ND	5
103		1328	1,1-diethoxy-2-phenylethane	fruity	MS, aroma, RI	3	ND
107	1724	1155	1,2-dimethoxybenzene	medicinal, hazelnut	MS, aroma, RI	4	4
111	1754		2-phenyl-2-propanol*	unpleasant	MS, aroma	2	ND
112	1756	1066	dihydro-5-ethyl-2(3H)-furanone	coconut	MS, aroma, RI	ND	5
114	1768	1247	ethyl 2-phenylacetate	rosy, honey	MS, aroma, RI	ND	8
115	1790		1,2-dimethoxy-3-methylbenzene	rosy, floral	MS, aroma, RI	5	14
117	1801		2-phenylethyl acetate	rosy, floral	MS, aroma, RI	5	14
123	1858	1090	2-methoxyphenol	spicy, clove, animal	MS, aroma, RI	6	ND
126	1872	1354	ethyl 3-phenylpropanoate	fruity, floral, rosy	MS, aroma, RI	10	15

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Table V. (Continued). Aroma compounds in the neutral fraction III detected by GC/MS and GC/O on DB-Wax and HP-5 columns

odor No.	*RI_{Wax}*	*RI_{HP-5}*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
128	1916	1276	2-phenyl-2-butenal	cocoa, sweet, rum	MS, aroma, RIL	5	4
129	1946	1228	benzothiazole	smoky, rubber	MS, aroma, RI	9	7
131	1952	1195	4-methyl-2-methoxyphenol	smoky	MS, aroma, RI	9	7
136	2004	987	phenol	phenol	MS, aroma, RI	7	9
140	2026	1281	4-ethyl-2-methoxyphenol	clove, spicy	MS, aroma, RI	5	2
142	2033		3-phenyl-2-propenal	sweet, cinnamon, spice	MS, aroma, RI	6	4
145	2079	1082	4-methylphenol	phenol, animal	MS, aroma, RI	9	11
146	2088		3-methylphenol	burning	MS, aroma, RI	7	ND
151	2172	1181	4-ethylphenol	smoky	MS, aroma, RI	12	10
154	2214	1310	2-aminoacetophenone	shoe insole, mothball	MS, aroma, RI	3	2
157	2308		2,6-dimethoxyphenol	smoky	MS, aroma, RI	5	8
163	2559	1388	4-hydroxy-3-methoxybenzaldehyde	sweet, floral, vanillin	MS, aroma, RI	12	9
166		903	4-ethoxy-2-pentanone**	fruity, green	MS, aroma	5	3
178		1134	1-(5-methyl-2-furanyl)-1-propanone**	baked	MS, aroma	ND	10

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odor No.	*RI_{Wax}*	*RI_{HP-5}*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
182		1290	pentyl hexanoate	sweet, fruity	MS, aroma, RI	4	ND
183		1424	1-phenyl-1-hexanone[**]	rosy	MS, aroma	ND	8

[a] MS: Compounds were identified by MS spectra. Aroma: Compounds were identified by the aroma descriptors. RI: Compounds were identified by comparison to pure standard. RIL: Compounds were identified by comparison with retention index from the literatures. [*] tentatively identified on DB-Wax column. [**] tentatively identified on HP-5 column. ***: tentatively identified on DB-Wax and HP-5 columns. ND: not detected by GC/O.

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Table VI. Aroma compounds in the neutral fraction IV detected by GC/MS and GC/O on DB-Wax and HP-5 columns

odor No.	*RI_{Wax}*	*RI_{HP-5}*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
4	929		dimethyl sulfide	cooked onion, sulfur	MS, aroma, RI	4	8
12	1020		2-butanol	fruity	MS, aroma, RI	4	ND
14	1035		1-propanol	ripe, fruity	MS, aroma, RI	ND	2
17	1061	764	dimethyl disulfide	onion, cabbage	MS, aroma, RI	4	1
19	1087	618	2-methylpropanol	wine, solvent	MS, aroma, RI	4	1
20	1114	703	2-pentanol	fruity	MS, aroma, RI	2	3
22	1137	643	1-butanol	fruity, alcoholic	MS, aroma, RI	3	ND
28	1201	783	3-methylbutanol	rancid, nail polish	MS, aroma, RI	13	13
37	1310	829	4-methylpentanol	fruity	MS, aroma, RI	2	3
39	1318	916	2-heptanol	fruity	MS, aroma, RI	4	ND
43	1334	815	ethyl 2-hydroxypropanoate	fruity	MS, aroma, RI	7	ND
45	1341	888	1-hexanol	floral, green	MS, aroma, RI	6	5
47	1350	923	2,3-dimethylpyrazine	baked, nut	MS, aroma, RI	8	ND
51	1382		3-octanol	green, fruity	MS, aroma, RI	ND	2
55	1400	1000	2,3,5-trimethylpyrazine	roasted, nut	MS, aroma, RI	7	ND
58	1413	950	4-methylhexanol	sweet, fruity	MS, aroma, RI	8	ND
59	1415	1030	2,6-diethylpyrazine	nut, baked	MS, aroma, RI	ND	6

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odor No.	*RI$_{Wax}$*	*RI$_{HP-5}$*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
65	1443	984	1-heptanol	fruity, alcoholic	MS, aroma, RI	6	2
66	1445	1088	2,3-dimethyl-5-ethylpyrazine	baked	MS, aroma, RI	6	9
69	1460	1093	2,3,5,6-tetramethylpyrazine	roasted, baked	MS, aroma, RI	12	12
73	1509	1163	2,3,5-trimethyl-6-ethylpyrazine	baked	MS, aroma, RI	7	ND
76	1527	1068	ethyl 2-hydroxyhexanoate	floral, jasmine	MS, aroma, RIL	8	13
77	1532		3-(1-methylethyl)-2-methoxypyrazine*	peasy, beany	aroma, RIL	6	10
78	1539	1074	1-octanol	fruity	MS, aroma, RI	2	2
81		1011	1-(2-furanyl)-1-propanone**	fruity, sweet	MS, aroma	7	ND
82	1562	1208	2-methoxy-3-butylpyrazine*	vegetable-like	aroma, RIL	13	5
83	1572	967	5-methyl-2-furfural	green, roasted	MS, aroma, RI	13	5
89	1593	1041	2-acetyl-5-methylfuran	roasted	MS, aroma, RI	10	ND
98	1643		1-nonanol	green	MS, aroma, RI	6	3
99	1647	854	2-furanmethanol	baked	MS, aroma, RI	4	3
102	1676	1357	3,5-dimethyl-2-pentylpyrazine	nut, baked	MS, aroma, RIL	8	4
105	1715	1249	2-furaldehyde diethyl acetal*	fruity	MS, aroma	8	ND
106	1722		1-phenyl-1-propanone*	fruity	MS, aroma	ND	2
110	1728		2,3-dimethyl-Z-5-propenylpyrazine*	baked	MS, aroma	11	10

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Table VI. (Continued). Aroma compounds in the neutral fraction IV detected by GC/MS and GC/O on DB-Wax and HP-5 columns

odor No.	*RI_{Wax}*	*$RI_{HP\text{-}5}$*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
112	1756	1066	dihydro-5-ethyl-2(3H)-furanone	coconut	MS, aroma, RI	5	5
115		1256	1,2-dimethoxy-3-methylbenzene	rosy	MS, aroma, RI	7	ND
118	1810		1-phenyl-1-ethanol	rosy, floral	MS, aroma, RI	ND	7
123	1858	1090	2-methoxyphenol	spicy, clove, animal	MS, aroma, RI	7	4
124	1862		geosmin	mushroom, earthy	MS, aroma, RI	ND	5
127	1906	1116	2-phenylethanol	rosy, honey	MS, aroma, RI	6	10
129	1946	1228	benzothiazole	smoky, rubber	MS, aroma, RI	4	4
131	1952	1195	4-methyl-2-methoxyphenol	smoky	MS, aroma, RI	ND	15
134	1959		Z-whiskylactone	coconut	MS, aroma, RIL	ND	12
135	1967	1024	2-acetylpyrrole	herbal, medicine	MS, aroma, RI	ND	3
139	2018	1363	dihydro-5-pentyl-2(3H)-furanone	sweet, cocoa	MS, aroma, RI	4	2
140	2026	1281	4-ethyl-2-methoxyphenol	clove, spicy	MS, aroma, RI	5	ND
145	2079	1082	4-methylphenol	phenol, animal	MS, aroma, RI	10	14
147	2113		4-propyl-2-methoxyphenol	smoky, phenol	MS, aroma, RIL	8	ND
149	2172	1181	4-ethylphenol	smoky	MS, aroma, RI	10	14
150	2180	1464	dihydro-5-hexyl-2(3H)-furanone	sweet, coconut	MS, aroma, RI	15	15

odor No.	RI_{Wax}	$RI_{HP\text{-}5}$	aroma compounds	descriptor	basic of identification[a]	intensity	
						MT	NJ
156	2348		4-(4-methoxyphenyl)-2-butanone	sweet, fruity	MS, aroma, RI	12	ND
157	2388		dihydro-5-(Z-2-octenyl)-2(3H)-furanone*	sweet, coconut	MS, aroma	10	11
161	2559	1388	4-hydroxy-3-methoxybenzaldehyde	sweet, floral, vanillin	MS, aroma, RI	2	3
167		938	3-methylhexanol**	green, fruity	MS, aroma	ND	4
170		957	ethyl 3-hydroxy-3-methylpropanoate**	fruity, sweet	MS, aroma	ND	4
179		1178	2-phenyl-1-propanol	fruity	MS, aroma	ND	8

[a] MS: Compounds were identified by MS spectra. Aroma: Compounds were identified by the aroma descriptors. RI: Compounds were identified by comparison to pure standard. RIL: Compounds were identified by comparison with retention index from the literatures. * tentatively identified on DB-Wax column. ** tentatively identified on HP-5 column. ND: not detected by GC/O.

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Table VII. Aroma compounds in the neutral fraction (fraction V+VI) detected by GC/MS and GC/O on DB-Wax and HP-5 columns

odor No.	RI_{Wax}	RI_{HP-5}	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
4	929		dimethyl sulfide	cooked onion, sulfur	MS, aroma, RI	2	ND
11	989	556	2,3-butanedione	buttery, cream	MS, aroma, RI	ND	4
17	1061	764	dimethyl disulfide	onion, cabbage	MS, aroma, RI	6	8
28	1201	783	3-methylbutanol	rancid, nail polish	MS, aroma, RI	8	10
40	1318		2-ethylpyrazine	baked	MS, aroma, RI	2	ND
42	1330	910	2,6-dimethylpyrazine	baked, nut	MS, aroma, RI	3	6
43	1334		ethyl 2-hydroxypropanoate	fruity	MS, aroma, RI	ND	4
47	1350	923	2,3-dimethylpyrazine	baked, nut	MS, aroma, RI	2	4
49	1375	981	2-ethyl-6-methylpyrazine	nut, roasted	MS, aroma, RI	5	8
55	1400	1000	2,3,5-trimethylpyrazine	roasted, nut	MS, aroma, RI	4	7
59	1415	1030	2,6-diethylpyrazine	nut, baked	MS, aroma, RI	6	ND
63	1429		2-hydroxy-3-hexanone*	fruity, berry	MS, aroma	6	2
64	1430	1080	2,5-dimethyl-3-ethylpyrazine	roasted, baked	MS, aroma, RI	ND	5
66	1445	1088	2,3-dimethyl-5-ethylpyrazine	baked	MS, aroma, RI	14	10
68	1456	831	2-furancarboxaldehyde	sweet, almond	MS, aroma, RI	ND	8
69	1460	1093	2,3,5,6-tetramethylpyrazine	roasted, baked	MS, aroma, RI	11	8
73	1509	1163	2,3,5-trimethyl-6-ethylpyrazine	baked	MS, aroma, RI	6	9

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odor No.	*RI_{Wax}*	*RI_{HP-5}*	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
82	1562	1208	2-methoxy-3-butylpyrazine*	vegetable-like	aroma, RIL	8	3
85	1583	1263	2,5-dimethyl-3-butylpyrazine	baked, roasted	MS, aroma, RIL	2	ND
86	1586	1075	ethyl 4-oxopentanoate	fruity	MS, aroma, RI	5	7
88	1592		2-(3-methylbutyl)-6-methylpyrazine*	baked	MS, aroma	11	ND
99	1647	854	2-furanmethanol	baked	MS, aroma, RI	8	4
102	1676	1357	3,5-dimethyl-2-pentylpyrazine	nut, baked	MS, aroma, RIL	4	6
110	1728		2,3-dimethyl-Z-5-propenylpyrazine*	baked	MS, aroma	4	5
112	1756	1066	dihydro-5-ethyl-2(3H)-furanone	coconut	MS, aroma, RI	5	4
115	1790	1256	1,2-dimethoxy-3-methylbenzene	rosy	MS, aroma, RI	14	ND
118	1810		1-phenyl-1-ethanol	rosy, floral	MS, aroma, RI	ND	7
120	1839		3,4-dimethyl-2-butenoic acid gamma lactone*	coconut	MS, aroma	ND	5
127	1906	1116	2-phenylethanol	rosy, honey	MS, aroma, RI	13	8
132	1955	1103	heptanoic acid	rancid, unpleasant	MS, aroma, RI	7	4
139	2018	1363	dihydro-5-pentyl-2(3H)-furanone	sweet, cocoa	MS, aroma, RI	4	2
142	2039		phenylpropanol	fruity, floral	MS, aroma, RI	ND	4
145	2079	1082	4-methylphenol	phenol, animal	MS, aroma, RI	5	5
147	2113		4-propyl-2-methoxyphenol	smoky, phenol	MS, aroma, RIL	3	4

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Table VII. (Continued). Aroma compounds in the neutral fraction (fraction V+VI) detected by GC/MS and GC/O on DB-Wax and HP-5 columns

odor No.	RI_{Wax}	RI_{HP-5}	*aroma compounds*	*descriptor*	*basic of identification*[a]	*intensity*	
						MT	*NJ*
148	2168	1268	nonanoic acid	unpleasant, fatty	MS, aroma, RI	ND	5
150	2180	1464	dihydro-5-hexyl-2(3H)-furanone	sweet, coconut	MS, aroma, RI	9	12
151	2204	1120	3-hydroxy-4,5-dimethyl-2(5H)-furanone	sweet, floral, spicy	MS, aroma, RI	ND	8
153	2220		4-vinylguaiacol	smoky, burning	MS, aroma, RI	9	ND
156	2348		4-(4-methoxyphenyl)-2-butanone	sweet, fruity	MS, aroma, RI	ND	4
157	2388		dihydro-5-(Z-2-octenyl)-2(3H)-furanone*	sweet, coconut	MS, aroma	8	8
159	2547		4-allyl-2,6-dimethoxyphenol	smoky	MS, aroma, RIL	5	ND
161	2559	1388	4-hydroxy-3-methoxybenzaldehyde	sweet, floral, vanillin	MS, aroma, RI	6	12
171		958	dihydro-5-methyl-2(3H)-furanone	coconut, fruity	MS, aroma, RI	5	ND
173		1012	2-methyl-6-vinylpyrazine	baked	MS, aroma, RIL	6	6
178		1151	dihydro-5-propyl-2(3H)-furanone	sweet, coconut	MS, aroma, RI	ND	10
185		1684	dihydro-5-octyl-2(3H)-furanone	coconut, sweet	MS, aroma, RI	4	6

[a] MS: Compounds were identified by MS spectra. Aroma: Compounds were identified by the aroma descriptors. RI: Compounds were identified by comparison to pure standard. RIL: Compounds were identified by comparison with retention index from the literatures. * tentatively identified on DB-Wax column. **: tentatively identified on HP-5 column. ND: not detected by GC/O.

Lactones

A total of 12 lactones were identified in this study. Most of lactones contributed to sweet and coconut aromas. γ-Decalactone had an aroma intensity of 15 in fraction IV (Table VI). γ-Heptalactone, γ-nonalactone, *Z*-whiskylactone, furaneo, sotolon, and dihydro-5-(Z-2-octenyl)-2(3H)-furanone (tentatively identified) were identified. Of these, *Z*-whiskylactone, furaneol, and sotolon were first detected in Chinese liquor. *Z*-Whiskylactone had a coconut aroma, furaneol gave strawberry aroma, and sotolon contributed to sweet, floral, and spicy aromas. γ-Butyrolactone, γ-pentalactone, γ-hexalactone, γ-dodecalactone, and 3,4-dimethyl-2-butenoic acid gamma lactone (tentatively identified) had low aroma intensity. The aroma thresholds of γ-nonalactone, γ-decalactone, and γ-dodecalactone were determined to be 91, 11, and 61 μg/L, respectively (*15*).

Other Compounds

2,4,5-Trimethyloxazole, 2-acetylpyridine, geosmin, 2-acetylpyrrole, geranyl acetone, and 1-ethyl-2-formylpyrrole (tentatively identified) were identified. 2,4,5-Trimethyloxazole was first detected in Chinese liquor, and it gave green, baked, and musty aromas (Table III). It was previously detected in acid-hydrolyzed and enzyme-hydrolyzed soy protein (*32*)., 2-Acetylpyridine, 2-acetylpyrrole, and 1-ethyl-2-formylpyrrole (tentatively identified), were also detected in this study. 2-Acetylpyridine contributed to popcorn and cooked rice aromas (Table III). 2-Acetylpyrrrole had herbacious and medicine aromas (Table VI). 1-Ethyl-2-formylpyrrole (tentatively identified) gave baked aroma (Table V).

Geosmin and geranyl acetone were also identified. Geosmin has been identified in Chinese liquor, and it gave musty, earthy and cooked-rice-husk-like aroma (Table VI). It causes an off-flavor to Chinese liquor at high concentration (*2*, *15*). This compound had a low odor threshold of 110 ng/L, and the concentration in Chinese liquors was reported from 1.10 μg/L to 9.90 μg/L, and from 1.04 μg/L to 3.79 μg/L in soy sauce aroma type liquors (*2*). Geranyl acetone gave rose and fruity aromas (Table IV), and intensity was 0~6. Its concentration in soy sauce liquor was 0~12.7 μg/L (*5*).

In summary, fractionation of volatile compounds using normal phase liquid chromatography coupled with GC/O and GC/MS is an effective technique to identify complex aroma extract. Esters, aromatic compounds, acids, pyrazines, and lactones were very important aroma-active compounds in soy sauce aroma type liquor.

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